

Ethical problems to merit the name

The new biology has spawned a new industry — that of ethical enquiries into the new biology. But the ethical problems are often neither new nor problematical. Hard heads and plain speaking are the urgent needs.

It is now universally accepted that novel techniques in biology create novel ethical problems, to which the scientific community and the world at large — not least those parts of it harbouring innate suspicions of innovation — have energetically bent their minds. In the United States, there is a host of committees and task forces worrying about the issues, many of them populated by people called “ethicists”. In Britain, the first study (in 1973) to anticipate most of the developments now exercising anxiety was carried out for the British Association by a committee under Professor (now Sir) Walter Bodmer for the British Association for the Advancement of Science (with the administrative and financial support of this journal). Since then, genetic manipulation has been regulated, the Warnock committee on embryo research and *in vitro* fertilization has laid the foundations of legislation (shockingly delayed) and now the Nuffield Foundation has set up a commission to take a more comprehensive view.

Much the same has happened elsewhere. In France, the government has created a standing commission to deal both with questions of principle and with issues as they arise. In Germany, various organs of the Bundestag and the government have a finger in the pie. Supranational bodies such as the European Commission are also likely to take an interest. Much enlightenment has come from many of these well ordered introspections, yet the question remains whether they are as necessary or as well-judged as their originators hope and claim.

The essential difficulty is the diversity of the problems occasioned by new techniques in biology. Organ transplantation depends on criteria for deciding when donors are dead; genetic manipulation is a potential hazard to the health of laboratory workers and may have environmental consequences if altered organisms are more generally released; *in vitro* fertilization raises questions about the meaning of, and respect for, human life; projects to produce the complete nucleotide sequence of the human genome raise the prospect that the outcome will be used both to discriminate unfairly between people already alive and to engineer the genetic health of those not yet born (eugenics is the codeword); interspecies relationships are held by some to be moral questions; and there are questions yet to be defined about the use that will be made of concepts of human individuality if ever it should be possible to transfer

information-content from one brain to another.

That many of these questions tax public understanding is relevant, but has not been a serious obstacle to enlightenment; the innumerable ethical committees have proved remarkably agile at coming to grips with unfamiliar problems. The findings of the committees are more often flawed by lumping the novel problems occasioned by the new biology together, regarding them as distinct from other, often older, problems.

There is a famous example in the report of the Warnock Committee, which recommended that the observation of developing human embryos should be allowed under licence, but only for a period of 14 days, whereupon the embryos must be destroyed on pain of criminal prosecution. The difficulty is that this compromise is different from that followed by British abortion law, which is supposed to rest on a diagnosed conflict of interest between the mother and her fetus, but which then allows abortion in the first two trimesters of pregnancy.

To be sure, the origins of compromise — better understanding on the one hand and a pregnant woman's interest *vis-à-vis* the fetus on the other — are not identical; in principle, the latter might well deserve precedence over the former. Certainly, at this stage there is no case for modifying the 14-day limit. But the two ethical arguments for compromise deserve to be weighed against each other, not to be tucked into separate compartments.

Much the same is true of the argument about the availability of personal insurance sparked by fears that insurance companies will take advantage of accumulating genetic knowledge to identify individuals carrying deleterious genes and then to deny them insurance, at least on normal terms. This is a deep question, but not ethically novel. Sadly, recognizable congenital defects — Down's syndrome, for example — are commonplace and those thus afflicted are at a disadvantage in the insurance market. So can there be an ethical distinction between an overt congenital defect and one that, until recently, has been cryptic? That distinction could be sustained only with the greatest difficulty, suggesting that some kinds of cruel bad luck are more deserving than others. Moreover, whether insurance companies are run as mutual funds or as public companies, those with the good luck (the presumed low risks) would quickly be



tempted away by other organizations if they belonged to a pool in which high-risk people were insured on the same terms.

But that does not mean that there is nothing to be said about insurance. Companies should not be allowed to walk away from contracts when previously cryptic information comes to light and should be prevented from making panicky decisions on the basis of a little genetic screening. But these are regulatory, not ethical questions. The outstanding question is an ancient one: that of whether society at large has an obligation to be the insurer of last resort to the otherwise uninsurable. It lies at the boundary not between ethics and biology, but between ethics and the politics of social welfare.

The eugenic issue is for the time being beyond the frontier of the possible, but is certain to be widely argued. Should people, individually, as couples or in groups, be encouraged or even allowed to use genetic information to improve the genetic quality of their offspring? Naive or malevolent ambitions of this kind (Galton and Hitler respectively) have rightly acquired a bad name, but we forget that most people are in a small way eugenicists. That, no doubt, is the biological intention of parents' frequent expressions of disapproval of their children's mates (often on the basis of spurious pointers — religious belief, for example). In the same spirit, careful parents attend to the quality of their children's diet. With the advent of genetic counselling and amniocentesis, it has become possible to avoid with a high degree of certainty the occurrence of particular genetic defects in the next generation (which may still carry the affected genes).

So would it not be preferable to manipulate the germ-line so as to remove unwanted mutations from extended families once and for all? What if it were feasible, for example, safely to replace the mutated by the normal globin gene in those carrying the sickle-cell anaemia trait? The standard answer, with which the scientific community seems to agree, is that the manipulation of the human germ-line, or even part of it for which a consenting couple may be responsible, is in all circumstances out of bounds. But can that position be reasonable? To be sure, those electing for such a procedure would have to be reasonably certain that there would not be a recurrence of world-wide malaria, so that the benefits of the relative immunity of heterozygotes for the sickle-cell anaemia substitution would not be lost; second-guessing the interests of future generations raises ethical questions, but questions already familiar in matters such as the provision of public education. But in principle (and if feasible) germ-line manipulation would seem to have the ethical edge over the practices now followed.

Not much of this argument is very new, but much of it is unpopular. Over the past few years, it has become politically correct to avoid the strongest arguments in favour of the accumulation and use of genetic knowledge for fear of giving offence — or of providing out-and-out

opponents of understanding with ammunition in their struggle against innovation. But in the long run, the outcome is a position weakened by what will later seem like misrepresentation. If, in a few years, there should be a strong case for observing embryos after 14 days, it will be more difficult to get the present British legislation changed than to have had it put in place at the outset. But the more serious weakness of the politically correct position is that it invests the new biology with an air of ethical daring that sometimes is inappropriate (as with genetic manipulation in the laboratory, which is a health and safety issue) and that often conceals long-standing political and social issues that merit attention in their own right). □

Third party assurance?

The Liberal Democrats in Britain have made a worthy but unconvincing pass at putting science to rights.

THE policies for British science laid out in the Liberal Democrats' Green Paper (consultative document) *Science and Survival* are surprisingly unimaginative for a party whose chances of victory in the next British general election are so small. While the party recognizes the hostility of the post-Thatcherite climate towards innovative science, its proposed solutions are themselves a little flaccid.

The green paper claims to see the efficient utilization of Britain's scientific resources as "the only way" of reversing the country's economic slide. Such a desperate and clear-cut situation, however, seems not to be desperate enough to have persuaded the party to the reinstitution of a science minister to oversee Britain's return to the top of the world's charts. "The cabinet", the group believes, "is already too large and . . . such a solution risks sweeping the issues that need to be addressed under the carpet."

Instead, the green paper proposes a cabinet committee, chaired by the prime minister, to "mastermind" the implementation of its policies. Essentially, these constitute a move away from the short-termism and general stinginess of present mechanisms for scientific funding towards a more "diffusion oriented" system, whereby innovation would be supported by industry, government and banks — all converted to appreciate the crucial role of science in the country's economic renaissance.

It is certainly a nice idea, as the policies of Liberal Democrats tend to be. It is also right and proper that it should begin with a strong argument that Britain needs a better educational system, but investment in education takes time to produce results; squaring that circle will not be easy. But this latest document lacks the conviction that might have earned the votes of the scientific community. The subtle distinction between being underfunded and being underpaid is one that the Liberal Democrats might with profit address if they ever decide to give these issues another shot. □

Trouble for Healy over misconduct office

- OSI turmoil prompts House hearing
- Healy's role in investigations probed

Washington

BERNADINE Healy, sworn in as director of the US National Institutes of Health (NIH) just over a month ago, is already the subject of bitter accusation and controversy, and this week faces a potentially bruising encounter with the House of Representatives oversight and investigations subcommittee chaired by Representative John Dingell (Democrat, Michigan). Dingell has evidence which — at face value — places Healy at the centre of a controversy involving the staple of NIH intrigue: scientific misconduct.

The storm centres on NIH's own misconduct detectives, the controversial Office of Scientific Integrity (OSI). Last month, Suzanne Hadley, former deputy director of OSI, resigned from the OSI's two flagship scientific misconduct investigations, citing interference by NIH in her work (see *Nature* 352, 268; 25 July 1991). This in itself would be enough to arouse Dingell's interest, as his subcommittee has vigorously pursued its own investigations into the same two cases: the alleged fabrication of data by immunologist The-reza Imanishi-Kari, and various issues centring on the work of Robert Gallo in the discovery of the AIDS virus.

But the revelation that a still-confidential OSI draft report, written by Hadley, would put Healy herself under OSI's spotlight means that this week's Dingell hearings will have added spice.

According to a source familiar with recent developments at OSI, the situation is this: Hadley began an investigation into alleged misconduct at Healy's old institute, the Cleveland Clinic Foundation, in December 1990. Her draft report found that a Cleveland Clinic biochemist had made false claims in applying for a series of NIH grants, one of which, approaching \$1 million in value, was awarded. Hadley's findings contradicted a preliminary internal inquiry at the clinic in May 1990, chaired by Healy, which had given the researcher the benefit of the doubt.

Because OSI reports can include an examination of any suggestion of a cover-up of misconduct, the final OSI report on the Cleveland case could be critical of Healy's handling of the initial Cleveland inquiry. The fact that Healy's husband, Floyd Loop, is chief executive officer at the Cleveland Clinic further complicates her position. In a statement released on Monday, Healy said that

reports of her role in the Cleveland misconduct case contained "half truths". Pressed for an explanation, an NIH spokesman said the reports had failed to mention that Healy had instigated a second inquiry — which later ensured the clinic received no federal funds under the disputed grant.

Healy's critics do not suggest that she has directly interfered with the Cleveland misconduct investigation to protect her own reputation. But Healy's decision to embark on the reorganization of OSI so soon after arriving at NIH, opening herself to the accusation that she has persecuted the author of the draft report on the Cleveland case, at best raises grave doubts about her judgement, say congressional sources.

Healy announced last month at the first meeting of the Public Health Service's new advisory committee on scientific misconduct that she had already launched an 'administrative review' of the office. She told the committee that OSI should perhaps be split into two arms — one to gather evidence, the other to make the final judgement of whether misconduct took place.

It seems that Healy's review of OSI had, by then, caused Hadley's departure from the Imanishi-Kari and Gallo investigations. At, it is said, Healy's request, OSI director Jules Hallum had requested that Hadley return her files on the two cases. Meanwhile, NIH general counsel Robert Lanman had been asked by Healy to examine Hadley's notes of her conversations with Margot O'Toole, who first made the allegations against Imanishi-Kari, after Healy heard from a number of NIH colleagues that Hadley had become close to O'Toole. Shortly afterwards, Hadley decided that it was time to quit the investigations.

OSI has a history of controversy — and charges of being both too easy and too hard on researchers under investigation. In September 1990, for instance, it came under fire from a House committee for failing to find misconduct in the case of a Pittsburgh Medical School researcher who had not revealed his pharmaceutical industry funding in NIH grant applications; on the other hand, a Wisconsin court decided that OSI's procedures for investigating misconduct were illegal because they failed to ensure that the accused received full due process, which has led to OSI's current solicitation of comment on its procedures.

But congressional observers of NIH are amazed at Healy's decision to start shaking up OSI while the Cleveland misconduct investigation was still in the works, and before Hadley had completed the two reports she was still working on. Hadley had already left OSI for another NIH position in March this year, but had continued handling the sensitive Gallo and Imanishi-Kari investigations. Healy had only to wait for these reports to be finalized, and then she could have tackled the reform of OSI without exposing herself to the complaint that her quarrel was with Hadley.

In addition to Healy and Hadley, NIH legal adviser Lanman, OSI director Hallum, assistant secretary of Health and Human Services James Mason and NIH deputy director William Raub are being called to testify before Dingell's subcommittee this week. NIH insiders are looking closely for any divergence between Raub's testimony and that of Healy. Raub, during his tenure as acting director of NIH before Healy's appointment, is said by one NIH source to have been "a stalwart supporter" of OSI, and is said to have assured Dingell's staff that Hadley would remain in charge of the Gallo and Imanishi-Kari investigations until their completion.

Just how badly this week's hearings could damage Healy is uncertain. "At best, I hope that this hearing shocks Healy into the realization that she should be more careful," says one congressional source. At worst? "She'll be crippled for the rest of her tenure."

Peter Aldhous

BRITISH SCIENCE

We shall be publishing on 12 September *Nature's* Manifesto for British Science, which will be offered without charge to any one of the political parties contesting the next general election, and in the belief that it is an improvement on their own version.

Following publication, on Friday 13 September, there will be a public meeting at the Royal Institution, Albemarle Street, London W1 at which Sir Mark Richmond, chairman of the Science and Engineering Research Council, will be the principal speaker. The chairman will be John Maddox, editor of *Nature*. The meeting will start at 9.45 a.m. and end at 12.45 p.m. The political parties will be invited to send representatives, but there will be ample time for discussion.

Those wishing to attend should apply for tickets to Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF.

Environmental advice

Washington

SENIOR executives from a number of leading US companies and a handful of representatives from environmentalist pressure groups have been appointed to a new think-tank to help develop US environmental policy. The President's Commission on Environmental Quality reflects President George Bush's belief that environmental problems are best tackled by voluntary cooperation between environmentalist groups and polluters, rather than by strict regulations passed down from government — a view contested by many environmentalist activists. The group's main focus will be on finding ways to solve pollution problems while minimizing the financial burden faced by industry, but its members have been given a reasonably free rein to set their own agenda. P.A.

Overkill at Stanford?

Washington

ANXIOUS to reestablish its image as a responsible guardian of public funds, Stanford University last week unveiled a new system of accounting for the indirect costs of federal research grants.

The reforms aim to avoid the inclusion of inappropriate items under indirect costs (such as the well-publicized yacht and antique commode) by setting up separate accounts to handle these unallowable costs.

Representative John Dingell, whose House of Representatives subcommittee investigation uncovered the irregularities in Stanford's accounts, has already expressed scepticism over the reforms. But an independent panel chaired by Bobby Inman, a former deputy director of the Central Intelligence Agency, believes Stanford may now have tried too hard to appease its congressional critics. "The accounting tail may well now wag the research dog," they write, in response to the proposed reforms. P.A.

Fetal tissue research

Washington

THE House of Representatives voted last week to overturn the US Administration's ban on using federal funds to support research on tissue from human fetuses. But the 274:144 vote fell just short of the two-thirds majority needed to override an expected presidential veto of the National Institutes of Health (NIH) authorization bill, which carries the fetal tissue language. Since its enforcement in 1988, the fetal tissue research ban has been a thorn in the side of NIH. The long delay in appointing an NIH director after James Wyngaarden's departure in 1989 is attributed in part to the difficulty in finding a candidate prepared to agree, at least in public, with the Administration. P.A.

It's official: no more shuttles

Washington

BARRING a repeat of the 1986 Challenger accident, no more US space shuttles will be built. Speaking at Vandenberg Air Force Base in California last week, Vice President Dan Quayle signalled publicly for the first time that the US Administration intends to wind down the troubled shuttle programme, and rely on a new generation of unmanned launchers to launch future satellites.

National Aeronautics and Space Administration (NASA) officials are said to have been seeking funds to add to the current fleet of four shuttles, and to build on shuttle technology to develop future launch vehicles. Quayle said the existing four craft would continue to be used into the next century, but he indicated that the shuttle's role as a 'cargo carrier' to deliver satellites into orbit was over: "It makes no sense to use shuttle astronauts unless we absolutely have to." Restrictions put in place after the Challenger disaster already ban the shuttles from

WEATHER SATELLITES

NASA rebuked for GOES-NEXT

Washington

US National Aeronautics and Space Administration (NASA) deputy administrator James Thompson found himself in an impossible, but familiar, situation last week: before a group of angry congressmen, defending NASA's role in a project that is massively over budget, badly behind schedule and beset with technical difficulties.

Two House subcommittees were trying to untangle the factors that led to the current fiasco in the GOES-NEXT programme. This series of five geostationary weather satellites designed for the National Oceanic and Atmospheric Administration (NOAA) is at least three years behind schedule and more than \$500 million over budget (see *Nature* 352, 97; 11 July 1991). The blame, judging by last week's hearing, lies largely with NASA, which hired contractors to build the satellites.

According to Howard Wolpe (Democrat, Michigan), chairman of the investigations subcommittee, senior NASA officials are even now making "deceptive and misleading" statements about the GOES-NEXT programme. Thompson testified last week that a wiring problem with the first satellite's imager should be resolved within four months, but Wolpe's aides say that NASA's own programme manager has informed them that this will take between six and nine months.

Wolpe also produced documents at last week's hearing showing that NASA had

carrying commercial payloads.

Quayle said the United States would develop a new National Launch System, comprising a range of unmanned launchers, some of which could also launch manned missions. This programme will involve NASA and the Department of Defense, but it is not yet clear exactly what Quayle and the other members of the National Space Council have in mind. John Logsdon, a space policy analyst at George Washington University, expects some of the system's individual components to become apparent by the time of next year's presidential budget request.

Quayle's comments came shortly after yet another delayed space shuttle launch. The shuttle Atlantis was due to take off carrying a Tracking Data Relay Satellite on 23 July, but the failure of a computer controlling one of the craft's main engines has now put the launch back to the end of this week at the earliest.

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refused to convert the GOES-NEXT contracts to a fixed-price agreement in 1988 — a move that would have saved some \$500 million in cost overruns — despite the willingness of both NOAA and the contractors to do so.

Thompson said he was confident that the first GOES-NEXT satellite, originally planned for 1989, will be launched by late 1992, providing coverage at least as good as the existing GOES-7, which will begin to drift out of position around that time.

But Wolpe's staff believe NASA's optimism over the GOES-NEXT schedule is unrealistic. NOAA has yet to make a firm decision on how to fill the gap in case of further GOES-NEXT delays, but the favoured option seems to be negotiating with the European weather satellite agency Eumetsat to move the Meteosat-3 satellite, already over the Atlantic, further westwards to give full coverage of the United States. The alternative, borrowing a satellite now being built by the Hughes Aircraft Corporation for the Japanese weather service, but not needed by them until 1994, involves the added risk and expense of a satellite launch, says NOAA assistant administrator Thomas Pyke. He adds that NOAA is now holding discussions with NASA to make the space agency responsible for development costs in future weather satellite programmes, preventing a repeat of the GOES-NEXT cost overruns from eroding NOAA's limited budget.

Peter Aldhous

Space programme in disarray

Paris

THE Soviet space programme is beginning to crack under the strain of a national economy in deepening crisis, according to Soviet officials.

Once shielded by the military, showcase space programmes such as the Buran space shuttle and the Energia heavy-lift launcher can no longer count on the armed forces now that East-West relations are warming. The faltering attempts at decentralization and a free-market economy under Soviet President Mikhail Gorbachev are considered threats to a dynamic Soviet space effort. *Glasnost* and *perestroika*, it seems, will not necessarily be good news for the world's leading manned-space programme.

Yuri Rhyzov, chairman of the Science and Technologies Committee of the Supreme Soviet, or parliament, offered some provocative insights into these problems recently in Paris.

"The political prestige of the government was always based on space," said Rhyzov, who is also rector of the Moscow Aviation Institute. "In addition, fundamental sciences thrived in the past because of the weapons and space industry. Scientists argued that all this science and space was needed to create new weapons, new rockets and so on. But I have the feeling that the military today is less interested in space."

In particular, military authorities have said publicly that they have no use for Buran — a move that Rhyzov said raises questions about whether the shuttle will ever carry a crew into space.

Modelled on the US space shuttle, Buran was developed in the mid-1970s "as a classified programme to develop mostly military missions", Rhyzov said. It has flown only once, in November 1988, in an unmanned launch by the Energia vehicle. That was only the second flight for Energia, which has since remained grounded.

Rhyzov and other Soviet space officials visiting the West in recent months have said the Soviet economy is in such disarray that no one can predict when, or whether, Buran or Energia will fly again. "We are now talking about budgets for three and four months instead of annual budgets, to deal with the fact that the system is bankrupt," Rhyzov said. "It is an impossible situation."

The government had initially agreed to support at least two additional Buran flights — one unmanned, the other carrying a crew — but now all that Rhyzov will say of the shuttle programme is, "It has not yet been formally cancelled."

The Mir space station, in orbit since February 1986, appears relatively safe from Moscow's economic turmoil, at

least for now. One reason is that Mir has become a source of foreign earnings. A private Japanese television station paid \$12 million for an eight-day voyage to Mir by a Japanese journalist last December, and the French and German governments have struck similar deals for flights in 1992.

A major difficulty for the Soviet civilian space programme is that its budget is inseparable from the military space effort. The government's annual space budget is given to legislators in a lump sum, with space-science missions and sending cosmonauts to the Mir space station bound up with telecommunications and spy satellites.

Under Gorbachev's government restructuring in 1988, the Supreme Soviet was supposed to assume greater authority to review government spending, in the manner of legislators in the Western democracies. This is illusory, Rhyzov said, and the Supreme Soviet in many cases "is just a decoration".

Parliamentarians tried to create line items in the space budget, only to find that the government erased them, he said. "Just recently the government cut these lines without the agreement of the parliament. There is concern that in today's very difficult economic situation, space will be the next field where unexpected budget cuts will be carried out. It has already happened in fundamental science and research."

One of the most important developments in Soviet space in the coming years, Rhyzov said, will be the increased independence of the Russian Republic, where most space work is done. Cutting back on the labour-intensive space programme would mean throwing whole towns out of work. Ironically this, rather than the value of the missions themselves, is uppermost in the minds of many in the government when they consider making deep cuts in space funding.

Adding to all these complications is the fact that *glasnost* and *perestroika* have not yet shed much light on what the Soviet space programme actually costs. The problem goes beyond the ruble's nonconvertibility.

"A ruble given to a space programme, or a military programme, has a much greater weight than a ruble given even to social programmes," Rhyzov said. Whereas on the black market a ruble has a value of less than US\$0.05, a military or space-programme ruble "is equal to the dollar, and may be worth even more," he said. Rhyzov said he supported attempts at creating a free-market economy in the Soviet Union, but the first effects of the change have resulted in a brain drain of space expertise. **Peter B. de Selding**

Navy made to pay for civil research

Washington

It may not be the 'peace dividend' that US researchers had been hoping for, but next year could see a subtle shift of funds from the Department of Defense to support civil science. Apparently with the tacit approval of the White House, the Senate appropriations subcommittee that handles the budgets of the National Aeronautics and Space Administration (NASA) and the National Science Foundation (NSF) intends to make the US Navy pay some \$105 million towards NSF's Antarctic science programme. The move is one of a series of creative accounting measures designed to accommodate the more than \$2,000 million being spent in 1992 on the space station Freedom and minimize its effect on other scientific projects.

The money in question covers the cost of providing logistical support for the US scientists in Antarctica, and the continuing clean-up of US Antarctic bases to conform with recently tightened standards for environmental protection. Navy ships supply US Antarctic bases, and Navy pilots fly the NSF's transport planes, but the NSF has reimbursed the Navy for these costs in the past.

In last year's appropriations bill, the Senate included a similar proposal to shift NSF funding to the Navy, but Richard Darman, director of the White House Office of Management and Budget (OMB), ruled the measure out of order — apparently to avoid a precedent that could blur the distinction between defence and civil spending. So it surprised Washington budget watchers that this year OMB has raised no objections to the change. "It seems pretty clear that the Administration is giving it some support," says Joel Whitter, from NSF's legislative affairs division.

The apparent change of heart is generally attributed to the Administration's determination to keep the Freedom project on course, in the face of opposition from the scientific community as well as some members of Congress. The battle fought in the House of Representatives this year over Freedom's funding is expected to be repeated with even greater intensity in the coming years as the annual cost of the project increases.

The funding shift will require the approval of the Senate appropriations subcommittee on defence; assuming that it and the OMB raise no last-minute objections to the change, the exact amount of money to be bled from the Navy to support the NSF should become clear later this year, when the House and the Senate go into conference over the appropriations bill.

Peter Aldhous

Cetus and Chiron merge

Washington

AFTER almost a year spent courting a corporate partner, Cetus Corporation of Emeryville, California, announced last week its intention to merge with neighbouring Chiron Corporation in a transaction valued at approximately \$660 million. The merger is contingent upon the sale of Cetus's PCR (polymerase chain reaction) business to Hoffman-La Roche Holdings for a planned \$300 million.

The combined company will keep the Chiron name, which is best known for the discovery of the hepatitis C virus. The new Chiron, which will have a joint worldwide workforce totalling 1,400 employees, expects eventually to cut its staff by 10 per cent to eliminate overlapping functions and allow full integration of the two companies.

The company will focus on four key areas of research: diagnostics; biopharmaceuticals, with an emphasis on cancer drugs; vaccines; and ophthalmics. Together, Chiron and Cetus have 22 products in human clinical trials.

The former rivals complement each other's strengths. Cetus not only provides Chiron with an entry into the therapeutic market through its oncology business, but also offers access to its specialized marketing and sales force both in the United States and through its European arm, EuroCetus. In addition, Chiron will be able to accelerate product development by tapping into the excess capacity of Cetus' manufacturing facility. "That's

worth a lot of money to us", says Rutter. "We [Chiron] will use manufacturing as a competitive advantage now."

With a combined cash balance of \$600 million after completion of the merger, which includes the \$300 million payment by Roche for PCR, it is unlikely that the new company will have to "worry about the short-term vicissitudes of the financial markets", says Ron Cape, chairman and chief executive officer of Cetus.

Cetus was dealt a severe blow in July last year when a US Food and Drug Administration (FDA) advisory committee failed to recommend approval of its key product, interleukin-2 (Proleukin), for the treatment of metastatic kidney cancer, even though no alternative treatment was available. The same product is approved for sale in nine European countries. This caused a rapid reversal of fortune, causing a precipitous drop in share prices and triggering a management shake-up after Robert Fildes quit the top job as chief executive officer in August 1990. In the ensuing months, Cetus embarked upon a series of cost-cutting measures in an attempt to curb operating expenses and win back the confidence of Wall Street and its investors. All of this fuelled speculation that Cetus was ripe for a takeover bid.

Margaret McGeorge, an analyst with Sutro and Co., says that from Chiron's standpoint it is an "opportunistic acquisition" as the company is not paying a huge premium for Cetus.

Robert Kupor, an analyst with Kidder Peabody, was less enthusiastic. Whereas most biotechnology companies are fairly conservative and stick close to their core technology, Kupor says that Chiron has shown the greatest tendency towards "empire building". He estimates that the crossover point when the new Chiron will be making as much as the old Chiron could be as far off as 1994 or 1995. "In other words, too far out for people to have any confidence in financial models," Kupor says.

News of the merger met with a cool response on Wall Street. Cetus's stock dipped 2.25 to 15.615 and Chiron's plunged 6 points to 54.75 on the day the news broke, amid concern among financial analysts that the merger could stifle Chiron's earnings momentum. As biotechnology companies go, Chiron has enjoyed considerable financial success and is in its fifth consecutive quarter in the black. By contrast, Cetus's losses for fiscal year 1990 totalled \$61 million.

Under the agreement, Chiron will exchange three-tenths of a share of newly issued Chiron stock for each share of Cetus stock. The ratio will be adjusted if Chiron stock tops \$67 per share or dips below \$49 over a fixed period of time. In addition, Chiron will assume \$145 million of Cetus' outstanding debt.

In addition to various regulatory clearances, the merger is subject to a majority approval by both Cetus and Chiron stockholders in meetings scheduled for early November. The two companies expect to merge by the end of the year.

Diane Gershon

Is Cetus selling the family silver?

Washington

It was only five months ago that Cetus emerged victorious from a federal court hearing in San Francisco with secure basic patent rights to polymerase chain reaction (PCR) technology, after a challenge by Du Pont. Now Cetus plans to sell its PCR business lock, stock and barrel to Hoffman-La Roche for \$300 million plus royalties. PCR — a technique for rapidly amplifying DNA *in vitro* — has revolutionized molecular biology since its development by Cetus in 1985 and has found broad application in the detection of genetic disorders, cancer and infectious diseases, paternity testing and forensic analysis.

While some might see spinning off the PCR division to Roche as selling the family silver, the deal actually seems to make sense for both Cetus and Roche. On the one hand, Cetus has already licensed most of the practical rights to PCR technology. On the other, Roche is developing and com-

mercializing *in vitro* human diagnostic products and services that are based on PCR technology for diseases such as AIDS, Lyme disease and tuberculosis. (And, through the Perkin-Elmer Cetus Instruments joint venture, in which Perkin-Elmer has a 51 per cent stake and Cetus owns 49 per cent, PCR technology is being used to develop instrumentation for the research and industrial markets.)

Under the new agreement, Roche will acquire ownership of PCR technology from Cetus, a transaction that includes all the technical, patent and manufacturing rights relating to PCR, although Cetus will retain the right to use PCR technology in connection with its therapeutic research. The Cetus and Perkin-Elmer joint venture will be dissolved and a new strategic alliance between Roche and Perkin-Elmer will be set up.

Ron Cape, chairman and chief executive officer of Cetus expects that the commercialization of PCR technology

will be more dynamic under Roche than it could have been under Cetus' control.

"PCR is worth more to Roche than it is to us", he says, particularly as most of the profitability with PCR is expected to be in clinical diagnostics. "Roche made us an offer we literally could not refuse", Cape says, and so Cetus opted for the cash in hand rather than a steady stream of future income.

Clearly, Roche is "willing to pay a premium to have full control of that business", says Jim McCament of the Medical Technology Stock Letter. "It makes perfect sense that they would buy the technology that they already have rights to", says Margaret McGeorge, an analyst with Sutro & Co.

With the sale of the PCR business goes Cetus' PCR division, which generated revenues of almost \$21 million in fiscal year 1990. Most of the division's 125 researchers are expected to move over to Roche's PCR facility in Alameda, California.

D.G.

BIOTECHNOLOGY

A first step, but still a long way to go

San Diego

THE first US Environmental Protection Agency (EPA) approval of a genetically engineered biological pesticide has heartened the biotechnology industry, but no one expects the decision to herald a flood of approvals for other bioengineered products. The pesticide was produced by a method that sidesteps many of the concerns about genetic engineering that have slowed regulatory acceptance.

In late June, Mycogen Corporation of San Diego received federal approval to begin commercialization of two pesticides, MVP and M-Trak, whose active ingredient is a protein, Bt, produced by the *Bacillus thuringiensis* bacterium. The successful conclusion to the six-year struggle to get approval brought smiles to many in the biotechnology industry, which has been frustrated by exhaustive regulatory restrictions.

Mycogen's president and chief executive officer, Jerry Caulder, characterized the attitude among his peers as, "Hey, we have one more notch on our gun. We're moving forward. It can be done and someone has done it."

Bt, which has been available as a pesticide for 30 years, is toxic to a variety of insects but harmless to humans. However, because it degrades within a few days, Bt's effectiveness has been limited. Mycogen improved this benign pesticide by inserting the gene coding for the toxic protein into the bacterium *Pseudomonas fluorescens*.

Mycogen avoids the questions surrounding environmental release of a genetically modified bacterium by a simple expedient. Following mass fermentation, the *Pseudomonas* bacteria are killed and treated with a patented process that stabilizes the cell walls and encapsulates the protein crystals. With such a protective shell, the toxins can persist in the field for up to eight days, Mycogen claims. And because no live bacteria are released, the company trimmed the number of years spent seeking federal approval.

Even with killing the bacteria, however, Mycogen was still required by the EPA to field-test its product rather than rely on existing data on Bt. Environmental groups such as the Environmental Defense Fund convinced the federal agency that the protein might be expressed differently in the new bacteria.

Other biotechnology companies are working on different ways to use Bt as a pesticide. Monsanto has created strains of corn that incorporate the Bt gene into the plant itself. Crop Genetics International is working on a plant 'vaccine' — seeds are inoculated with a bacterium carrying the gene for the Bt protein.

Judy Berlfein

US NATIONAL LABORATORIES

Staying with California labs

Washington

THE US Department of Energy (DOE) plans to renew its contract with the University of California to manage the Lawrence Berkeley, Lawrence Livermore and Los Alamos national laboratories. The decision, announced by Secretary of Energy James Watkins on 24 July, ends ten months of speculation that the DOE might bring in another contractor, probably from the private sector, to run Livermore and Los Alamos — which together are home to most of the US government's nuclear weapons research.

Last week's announcement pleased most researchers at the laboratories, who jealously guard the academic freedom they are allowed within a university-managed setting. During the Gulf War, this became a source of friction between the university and DOE, with the department said to be annoyed at the freedom with which Livermore and Los Alamos researchers were allowed to talk to the media.

The decision will be less pleasing to many on the faculties of the various University of California campuses, which have long been uneasy with the system's association with military laboratories.

There was widespread speculation that DOE was not completely satisfied with the California system, either, and that it would open the Livermore and Los Alamos management contracts to competitive bids from a range of organizations. Watkins has expressed a desire to make DOE contractors responsible for any costs incurred because of lax environ-

mental standards at the facilities they manage — something the University of California was unwilling to do. And last year came revelations that tens of millions of dollars worth of equipment and some government documents had been found to be missing from Lawrence Livermore (see *Nature* **346**, 97; 1990).

Last September, the university's governing board of regents decided to negotiate to renew the management contracts despite opposition from faculty. But California officials had since made it clear that they are not interested in competing for the contracts.

In agreeing to negotiate on a new contract, the DOE seems to have agreed not to insist that the university system accept financial liability for environmental damage caused by the laboratories.

Some university officials speculate privately that Watkins may have decided to leave the national laboratories with California some time ago, but delayed his announcement to avoid the impression of caving in on the environmental liability issue. Watkins is said to be determined to bring the most polluting among DOE facilities, notably the nuclear weapons production plants, to heel.

The University of California's continued management of Lawrence Berkeley was never in serious doubt, because of the laboratory's intimate association with the university's Berkeley campus. All three laboratories have been run by California since their inception as national facilities between 40 and 50 years ago.

Peter Aldhous

INDIA

Meagre science budget

New Delhi

A crisis in both resources and foreign exchange has forced the two-month-old Indian government to set a meagre 1991-92 budget of 23,900 million rupees (£570 million) for scientific research and development activities not relating to defence. This is about 2.6 per cent of the country's total budget as it was presented to parliament last week.

Although on paper the research and development budget is nearly 12 per cent more than was spent last year, the increase is apparent rather than real. With the rupee having been devalued since last year by nearly 25 per cent against the pound, the various scientific agencies — which import most of their equipment from abroad — will actually have less to spend.

This year's budget has no allocations for new projects in any of the scientific

agencies except the Department of Space. That department plans to set up a third launch pad on the east coast for launching polar satellites and is also hoping to build a new satellite dedicated to beaming educational programmes to classrooms. The government has approved these two schemes, but has made only a token provision of £80,000 in the budget.

After announcing a new industrial policy that makes it easier for the entry of foreign capital and technologies, the government intends to overhaul science and technology policy as well. Finance Minister Manmohan Singh said that science and technology policy will be reoriented in order to relate it "more intimately to the requirements of our development as well as for better absorption and assimilation of new technologies".

K. S. Jayaraman

Questions at Sellafield

London

OF all the possible locations in Britain, a government contractor has chosen the ground beneath the Sellafield nuclear facility as the best geological site to store the plant's radioactive waste. UK Nirex, the nuclear waste management firm, last week ended an eight-year search for a suitable site by picking Sellafield over an alternative location at another nuclear facility in Dounreay for a £3,000 million national nuclear waste depository.

Nirex admits that it does not know if the Sellafield site is actually the best in Britain, nor, for that matter, does it intend to find out. Officials decided early in the search processes simply to find the most attractive site that could meet international safety guidelines for such depositories. After completing two of a planned six boreholes at each site, it decided that Sellafield's geology would pass muster. Once that was established, Nirex concluded that the estimated cost savings of £1,000 million in not having to transport the waste off the Sellafield site was reason enough to select it for the depository, and to suspend the geological analysis of the other site.

Some UK geologists are bothered that economics finally won the day. "We were concerned that the decision was premature when one looks at it from a technical point of view," says Adrian Bath of the

British Geological Survey, which did some of the research on the sites for Nirex. He says it is not yet possible, on the basis of two boreholes at each site, to distinguish which of the two final sites is better from a geological point of view. "At the end of the drilling programme when you have some 10 boreholes, then you can make a distinction," he says.

Current plans call for three 800-metre deep caverns, each 250 metres long and 35 metres high, which are eventually to hold between 700,000 and 2 million cubic metres of waste.

Last week, UK environmental groups criticized the decision, and Greenpeace released a report on the known data about the proposed sites.

The report, by independent geologist Philip Richardson, concludes that Nirex's analysis of Dounreay was based on "grossly oversimplified geological interpretation", and that the Sellafield site was characterized by "totally the wrong [in this case, highly fractured] rock type." The report concludes: "there can be no confidence in an exercise which, having searched the whole UK mainland and continental shelf, identifies two nuclear sites as the best candidates for a repository." Nirex narrowed the search to two sites in 1989 after considering some 500 other UK prospects.

Christopher Anderson

INTERNATIONAL LABORATORIES

UK backs out of neutron lab

London

OFFICIALS at the troubled international neutron laboratory at Grenoble, which was shut two weeks ago after cracks were discovered in part of its reactor cooling system, received another dose of bad news last week: Britain announced that it intends to cut its funding for the centre by nearly half.

The council of the UK Science and Engineering Research Council (SERC) decided earlier in July to seek to renegotiate the current UK two-year contract with the Grenoble laboratory, known as the Institut Laue-Langevin (ILL). Rather than maintain a one-third partnership with France and Germany in the laboratory, SERC wants to reduce its commitment from £11 million to about £6 million annually by 1994. SERC's £22 million neutron research budget, currently split evenly between ILL and the ISIS neutron facility at the UK Rutherford-Appleton Laboratory, is to be reduced to £17 million for the 1993-94 budget. But the SERC council declared its "unambiguous commitment" to ISIS and placed it off-limits at least through 1997, leaving ILL

as the only place to cut.

ILL officials say that the shortfall could be made up by bringing in another partner (Italy is rumoured to be a possibility) or by asking the other two current partners to increase their contribution. Germany is strongly in support of the facility and is prepared to discuss various solutions for upgrading the facility, says Anne-Marie Hanson, the official in charge of ILL at the German Ministry of Research and Technology.

In the meantime, officials hope that the UK decision will not interfere with plans to repair the reactor, a process that is expected to take at least two years (see *Nature* 352, 178; 18 July 1991). "The [decreased] British contract wouldn't start until in 1994," says ILL director Peter Day. "We're talking about [repairs] that might be proposed in the next few months."

At the Rutherford-Appleton Laboratory, ISIS officials have requested two extra weeks of beam time in order to accommodate some of the researchers stranded by the ILL shutdown.

Christopher Anderson

JAPAN

Neuroscience leads grants

Tokyo

NEUROSCIENCE features prominently in Japan's biggest grants for individual scientists, announced last week by Japan's Ministry of Education, Science and Culture. The boost for neurological research reflects increasing government concern about Japan's ageing population and the associated occurrence of neurological disorders among older Japanese citizens.

The ministry's 'special distinguished' grants, which are worth about US\$1-2 million for 3-5 years, are meant to go to internationally recognized researchers and usually give a good indication of where Japan's best academic research is being carried out. But defects in the grant screening process occasionally lead to odd choices, and so the awards should always be interpreted with care.

This year's 12 awards include three in the field of neuroscience. Toshiharu Nagatsu at the School of Medicine of Fujita Health University gets ¥205 million (\$1.5 million) over three years to carry out molecular genetic studies on the structure, function and pathology of catecholamine neurons, which play important roles in the higher functions of the central nervous system.

Similarly, Yasuo Ihara at the Institute of Brain Research at Tokyo University, was awarded ¥105 million over three years to examine modifications of a microtubule protein called tau that occurs in the fibrous structures associated with Alzheimer's disease. And Shigetada Nakanishi of Kyoto University, gets one of this year's biggest grants — ¥237 million over four years — to study the function and regulation of glutamate receptors, which play a key role in memory acquisition and learning.

Japan is one of the most rapidly 'greying' nations in the developed world. A postwar baby boom followed by a plunging decline in birthrate means that the population will be dominated by elderly people early in the next century. As a result, pharmaceutical companies and the government are pouring investment into the development of cures for neurological disorders associated with old age.

Among other grants of practical interest is one to Masayoshi Esashi of Tohoku University to make microactuators using photofabrication techniques. This project, which will be funded with ¥232 million over four years, forms part of a steady push by Japan to develop micromachines and nanotechnology. And Jun Akimitsu of Aoyama-Gakuin University gets ¥180 million for three years to find new high-temperature oxide superconductors.

David Swinbanks

Manning the cannons at ICI

London

"I've seen no bid. Maybe you know something I don't." This is how Sir Denys Henderson, chairman of Imperial Chemicals Industries (ICI), waves away a reporter's questions about a threatened takeover. Over at Hanson PLC, from where the assault is supposed to come, the same question draws blank stares. "Bid? I have no idea what you are talking about," a spokesman says, deadpan.

For three months now, Britain's largest research corporation and its most notorious company-acquiring conglomerate have maintained this straight-faced charade. Despite Hanson's menacing purchase of nearly three per cent of ICI in May, the notorious corporate raider has so far made no public move to take the rest of the company, beyond a standing offer of an amicable merger.

Nevertheless researchers, some 13,000 of whom work for ICI or collaborate directly with its scientists, have watched the manoeuvring nervously. A takeover attempt could mean substantial cuts in research, either to raise money to thwart the bid, or, if Hanson is successful, to generate short-term profits for the bottom-line obsessed conglomerate.

Last week, 23 leading academics and members of the Royal Society signed a letter to the London *Times* airing their fears about a takeover. "Hanson is good at its own business, but it has far less experience in running a research-intensive business like chemicals," they wrote, citing the fact that ICI spends about £679 million (5.3 per cent) of its total sales on research, while Hanson's research and development budget (totalled over the nearly three dozen companies that make up the conglomerate) is only some £34 million (0.4 per cent) of total sales.

Like its US rival Du Pont, ICI is primarily a chemicals company, although it also has large pharmaceutical and agriculture arms. It is the company that invented polyester, polyethylene and beta blocker heart drugs, as well as being the first to launch both a CFC replacement and a biodegradable plastic. In pharmaceutical, it now concentrates on cancer and cardiovascular fields, and has six anti-cancer drugs in the pipeline.

ICI spends about £7 million on some 60 collaborations with UK academic scientists, and £2 million on joint projects outside Britain. Those links, the scientists warned in the letter to the *Times*, "could be quickly destroyed by short-term savings which significantly reduced research, thinned out technological capabilities and altered scientific collaborations that are essential for the long-term competitiveness of the company."

In fact, the very threat of a bid has already spurred ICI to trim research. Desperate for an encouraging quarter of profits, ICI decided earlier this year to abandon much of the high-performance materials business, where it had been hit by tough competition in the United States from General Electric and a peace-time decline in the aerospace industry, which uses the sort of high-temperature plastics and composites that ICI was developing. It has already begun to shut down most of its advanced materials research operation, a process that will eventually mean a reduction of £30-50 million in research and development and a loss of some 250-300 research positions.

But the worst may be over. Last week, ICI, which owes its vulnerability to a half-decade of diminishing profits, counterattacked with the first results of its promised £300 million restructuring: it announced profits that, while still one-

third lower than the first half of last year, were well above expectations in the midst of a recession. As the restructuring takes effect, Sir Denys promises even stronger performance in the months to come, something he hopes will restore shareholder confidence in its management and take the wind out of Hanson's sails.

The Hanson threat may actually have galvanized ICI to make some changes to improve the company's position in the years to come. Although cuts and streamlining are still in the cards, research as a whole appears likely to miss the axe. Since 1984, ICI has increased its research spending some 70 per cent relative to total sales, 30 per cent more than the average UK chemical company. Unlike some US companies that responded to takeover threats by selling research arms to generate cash, ICI "has no plans to sell off any R&D to fight a takeover bid," says research director Peter Doyle.

A study by the Science Policy Research Unit at the University of Sussex shows that ICI's research has steadily improved over most of the past decade, especially in comparison to its UK competition. Worldwide, the picture is not as encouraging, however; although ICI is among the top 20 companies in six US patent areas, it is well behind competitors such as Du Pont (top 20 in 14 areas) and Bayer (top 20 in 10 areas).

The trend, however, is on ICI's side. A study by CHI Research, a US innovation analysis company commissioned by ICI to evaluate its research and development, found that the ICI's technical strength, as measured by both the quantity and the quality of its patents, has increased from 3.77 in 1986 to 6.9 in 1989. Although that is still behind Dupont (12) and Bayer (9.7), it is well above the industry average of 1.3. And the current restructuring, while cutting costs across the board, is not expected to hit research as hard as other sectors. **Christopher Anderson**

Hanson: research versus the bottom-line

What do Jacuzzis, bricks, fish oil and easy-listening radio have to do with research? Not much, and that is what worries scientists most about Hanson's threatened takeover attempt of ICI.

Essentially, Hanson is a conglomerate with no special industrial focus. It owns more than 30 companies in the United States and Britain, ranging from construction and building industries to manufacturers of consumer products. Besides baths and bricks, its subsidiaries make such products as vitamins and concrete — basic goods that require little new technology.

By design, Hanson has acquired few companies that do research. The 'Hanson formula' is aimed at quick profits and accelerated product development, not

long-term projects. The corporate philosophy consists mostly of selling off or closing the least productive sectors of a newly acquired company and decentralizing management in the remainder to give local managers the responsibility for improving productivity. When Hanson does acquire a company that has a significant research component, its policy is to move research groups closer to production facilities, to ensure that research and development is focused on areas that will quickly turn out marketable products.

Perhaps the best example is British Ever Ready, the UK counterpart of the US battery manufacturer. After acquiring the company (then called Berec) in 1981, Hanson quickly sold its Abingdon-based

Advanced Projects Group to the researchers themselves. Hanson then moved a smaller Ever Ready research and development team from London to a battery factory in northeastern Britain.

One study estimates that Hanson might cut 15,000 jobs if it took over ICI, part of a fat-trimming exercise aimed at increasing profits at the chemical giant. Research would almost certainly suffer. On the other hand, Hanson has grown over ten-fold in the past decade; last year it had profits of £1,285 million on £7,153 million worth of sales. It had to make harsh cuts in the companies it acquired to get there, but many of them subsequently saw profitability for the first time in years. **C.A.**

Genome ethics treaty

SIR — The last decade of this century may well be remembered as the true beginning of the era of human gene diagnosis and therapy, but also as a time of confusion and confrontation over the ethical, legal and social implications of the applications of recombinant DNA technology to humans.

These divergences were patent at a conference recently held at the National Institutes of Health on ethical and social issues raised by the human genome project, which failed to reach conclusions about the need for a genome ethics treaty. Yet many participants urged the need for a consensus on fundamental questions. It may be too soon for a detailed code of behaviour, but supranational and national institutions are asking the scientific community for at least some unquestionable guidelines. And much could be done.

For example, in October 1988, what is called the *Declaración de Valencia* included some general statements proposed by the participants of a Workshop on International Cooperation for the Human Genome Project held in Spain. It says that "mapping and sequencing of the human genome should be only used to increase human dignity". It also urges international coordination on research and information, which should be in the public domain.

Although research should not be stopped, potentially hazardous experimental strategies should be strictly controlled — among them germline as distinct from somatic DNA intervention. The first issue implies a manipulation of the heritable genetic information

from all cells. This might explain the logical fears raised in Germany because of its potential use in Nazi-like eugenics policies¹.

On the other hand, nobody can deny the potential benefits of somatic DNA therapy, as proposed for example for cystic fibrosis by introducing the normal gene into lung cells². The first encouraging results of the applicability of gene therapy in other diseases have recently been demonstrated by *in vitro* infection of tumour-infiltrating lymphocytes with a retrovirus expressing a selectable marker and their reimplantation into donor cancer patients³. That study provides the first clinical data on the safety and efficacy of gene transfer in humans and points to its application to single-gene recessive disorders, where a correction of the endogenous mutant gene is not required.

It now seems appropriate to establish a reference code on somatic cell modification, but more information on the consequences of germline manipulation in experimental models is required before a code for its human application can be determined.

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Orange sky . . .

SIR — Your recent note about ball lightning (*Nature* **350**, 108–109; 1991) mentioned a few sightings by "qualified scientists". I am both a professional and amateur astronomer and an active storm chaser, and wish to describe my recent observation of bead lightning (multiple ball lightning?).

Some doubt is still expressed about the reality of this phenomenon. After-images from a bright flash, or chance alignment of lightning segments towards the observer, have been suggested as possible explanations, but the circumstance of my sighting denies these possible explanations.

On the evening of 17 February 1990, I stepped outside to examine the possibility of recording lightning on video. After a short time, there was a brilliant flash behind me and, on spinning round, I saw a 'zig-zag' trail of perhaps 30 small orange balls, quite evenly spaced, outlining the path of the lightning stroke. The balls looked of uniform size, with the higher balls being fainter and of smaller angular diameter. Towards the bottom

end, the balls were around half the angular diameter of the Moon.

Another sighting of the same lightning strike was made by people in another house down the street and gave a parallax of nearly 90°. (These witnesses saw no balls, presumably as they were significantly fainter than the lightning stroke.) This indicated a distance of 50 m from myself and gives an actual size of 15 to 25 cm for the balls. Examination of the garden and buildings where the strike would have grounded failed to show the precise spot, but the general area of the strike is consistent with damaged appliances in surrounding houses and the tremendous clap of thunder following rapidly after the strike.

The balls showed no motion and faded very rapidly, lasting perhaps 1 second, with no smoke or other remnant at their disappearance. In appearance they were roughly circular, orange on the outside, brightening towards the centre which I think was white. The integrated brightness of each ball was roughly magnitude –4, comparable to Venus and certainly much less than the brightness of the full Moon (–13). Despite daylight, it being 7 p.m., the sky was very dark with a total overcast.

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Bacon's chicken

SIR — Your correspondent W. F. J. Cuthbertson attributes the development of refrigeration for preserving food to the invention of the compression expansion refrigerator in 1872 (*Nature* **351**, 434; 1991). The history of food refrigeration is in fact considerably longer. The principle was certainly used domestically in Europe and North America in the eighteenth century, as shown by the icehouses of great estates in England and the then American colonies, and it was being exploited for the export of perishable goods from New England to Calcutta by 1833. Exports of ice from Boston exceeded 100,000 tons a year by the mid-nineteenth century¹.

Credit for the concept of food refrigeration must go to Francis Bacon, Viscount St Albans, whose experiment to preserve a chicken carcass using snow took place at Highgate in 1626. Bacon died of pneumonia three days later². The shelf-life of the chicken is not recorded.

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Sanctions on Iraq

SIR — The suggestions by Abdalla O. Elkhawad and Scott D. Cobourn (*Nature* **351**, 9; 1991) are not sensible or reasonable. To place a scientific embargo on the Iraqi scientific community would do no good because limiting science would only harm everyone. A better policy would be to establish or maintain contact with the Iraqi scientific community in order to exert influence within Iraq. As an example, Canada's foreign policy towards South Africa comes to mind. At no time did Canada ever sever relations with South Africa. Not only has it maintained an embassy in South Africa, it has also allowed the continued import of South African scientific periodicals and journals to Canadian universities and institutes, as well as permitting trade and other informal contacts to continue.

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Testing the science testers

Mary Jane Drummond

The new national curriculum for England and Wales includes the testing of the scientific ability of seven-year olds. But the tests themselves show evidence of scientific illiteracy.

DURING the summer term of 1991, a new and controversial provision of the 1988 Education Reform Act came into force. For the first time, teachers of six- and seven-year olds were required by law to assess their pupils' learning in mathematics, English and science by means of "Standard Assessment Tasks". These tasks were developed by the Schools Examination and Assessment Council, and are designed to measure every individual child's achievement against "attainment targets". These targets are laid down as part of a national curriculum, which prescribes what is taught in primary schools in the form of "programmes of study".

The question of how the statutory programmes are to be taught remains the professional responsibility of teachers; but the question of how children's achievements are to be measured is now governed by an act of parliament. The statutory testing programmes, to be administered to every child in England and Wales who reaches the age of seven during this academic year, comprise a set of classroom activities, or tasks, tightly specified in every detail. After administering and observing the tasks, teachers must assign each child's performance on each attainment target to one of three levels of achievement. The criteria of these three levels, and precise instructions for administering the tasks, are given in the *Teachers' Handbook* and

the *Assessment Record Booklet*, issued to every teacher involved by the Schools Examination and Assessment Council. During the first half of the summer term, regular classroom routines were set aside so that teachers could carry out this massive programme of testing.

Serious concern

The public debate about the new requirement has, in the main, centred on the question of whether such young children should be formally tested in this way; many educators have expressed grave anxiety about the possible harmful effects of assigning young children to one of three levels of achievement. What has been less widely discussed, but is, I believe, of equal importance, is the nature of the tasks themselves: the concepts that are being tested and the criteria for success. In particular, the one compulsory standard assessment task for science seems to me to need extensive revision.

The *Teachers' Handbook* says of this task: "This activity assesses children's process skills in observing, exploring and investigating a variety of characteristics and properties of real objects, and their skill in communicating their findings." This part of the science curriculum is the only so-called "process" target, that is concerned with the scientific process itself, rather than with aspects of scientific knowledge, which are covered by

other standard assessment tasks. It is, therefore, particularly important if a worthwhile assessment of children's scientific thinking is to be made.

The compulsory standard assessment task comprises two different tasks; the teacher is required to observe small groups of children carrying out these tasks, and then to judge whether each of them has reached level 1, 2 or 3. To assess whether a child has reached level 1, the teacher provides a collection of twenty natural objects and involves the children in a "describing game". Each child is asked to "use more than one sense to describe three objects", and "to describe three objects in terms of their physical characteristics". This might, in fact, be considered an assessment of a child's expressive use of language, rather than his or her scientific achievements, but more serious problems arise in the assessment of levels 2 and 3.

The activity here is entitled "exploring floating and sinking". The teacher is instructed to collect 16 "objects made of different materials". Each child being tested (in groups of four) is given four objects to include: "at least one object that has some features which are easier to see using a hand lens, for example, a twig, a stone or a piece of fruit; at least two objects that will float, for example, a twig and an apple; at least one object that will sink, for example, a stone; and at least one heavy object that will float, for example, a conker or a piece of fruit. All the objects must have differences in weight that are detectable, using classroom balance scales with standard or non-standard units." Each child is given a chart on which to record their findings — a simple grid with 16 cells, four to be completed for each object. The columns are headed: "What is it? How heavy is it? Do you think it will float or sink? (PREDICT) Did it float or sink? (TEST)". The children are required to draw, weigh, predict and then test each object "to find out as much as they can about why some objects float and some sink."

First, the drawing. The instructions are very precise: "Explain to the children that they should observe and begin to draw each object without using a hand lens and then finish the detail of their drawing of each object with the use of a hand lens. Throughout the activity, you



A worthwhile assessment of scientific thinking?

can prompt the children when to use, and when not to use, the hand lens." The use of the hand lens is evidence for the achievement of level 3, for which the criterion is "select and use simple instruments to enhance observations."

Scientific thinking

Readers (or seven-year-old children) may not immediately see the connection between enhanced observation of the exterior of an object and whether it will float or sink. But the instructions to the teacher cover this point: "Make it clear to the children *why* they are drawing each object. The drawings are a record of their observations to see if there is anything about the appearance of each object, for example texture, that *may* cause it to float or sink". In my hearing, one teacher expressed this instruction to the children as: "Look carefully at your objects to see if there's anything that will affect how they float or sink," and another: "Look to see if there's anything on the outside that might help it to sink or float." A child examining a wood-shaving was instructed: "See if you can see anything on it. . . ." and replied "little lines?" The teacher asked "Will the little lines help it float?" The child was, understandably, silent.

Next, the weighing: the child is to "find and record the weights of three of the four objects by comparison to standard or non-standard units using balance scales. Comparison using 'feel' is not sufficient." This part of the task might be considered as a test of a child's understanding of the use of standard weights and, when the child has weighed, for example, a coconut, with assorted gram weights, of the child's ability to total correctly a string of different numbers up to anything between 500 and 1,000. But it has in fact been designed as part of a test of scientific thinking.

The assessment of the child's ability to predict and hypothesize is even more questionable. The *Teacher's Handbook* instructs: "Ask each child to tell you what they think will happen if one of their objects, which you name, is placed in the container of water. Then ask *why* they think this will happen. With each child, repeat this with a second object. The purpose of this questioning is to see if the child can predict happenings or hypothesize." The *Assessment Record Booklet* amplifies these criteria: "correctness of prediction is not important" and "a reasoned explanation. . . is an 'I think. . . because'-type statement. The relationship does not have to be 'correct'". So, the ability to predict, in its scientific sense, is established by the ability to supply one of two words ("float" or "sink") in response to the teacher's question. Either word will do,

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Looking at how objects float and sink.

whatever the object: a child who predicts that a brick will float shows the same evidence of attainment as a child who predicts that it will sink. And the ability to hypothesize, using reasoning, is established by the ability to frame a sentence about floating and sinking using the word "because". A child who reasons that an orange will float (or sink) because it is orange, or smooth, or dimpled, shows the same evidence of attainment as a child who reasons that a log will float because it is made of wood and wooden things float.

Generalization

Next, children are invited to give evidence of other attainments: "When asked what they have found out for any *one* object, they are able to link an observed and recorded characteristic with floating or sinking, for example weight, colour, texture. *The relationship does not have to be 'correct' but must be based on their findings.* Can express their findings as a *generalised statement.* The statement must apply to *more than one* object that the child used in the investigation, for example, the heavy things sank, the large things sank, some heavy things sank and some heavy things floated, the smooth things floated. *The generalisation does not have to be 'correct' but must be consistent with their observation.*" So a child who generalizes 'all the orange objects floated' is judged to be at the same level of scientific exploration as a child who generalizes that 'all the things with air in floated'. It is not surprising, perhaps, that one child, when asked what he had found out, replied: "I *think* I've found out that heavy things float, and light things sink. But I know that can't be right."

I believe that there are several serious flaws in this approach to assessing children's scientific thinking. The floating

and sinking task was specifically designed to assess the process that the national curriculum labels "exploration of science". I have no reservations about the intrinsic value of this process, and I have no doubt that the vast majority of six- and seven-year olds can give convincing evidence of their ability, and willingness, to explore science. But I do object to the dangerous weaknesses in the scientific process embodied in this task. In particular, I object to the use throughout the test of the terms "heavy" and "light" as if they were absolute, not relative; the suggestion that "why some objects float and some sink" can be investigated by drawing, using a hand lens and weighing; the confusion of the nature of scientific prediction with the ability to complete a sentence with one of two words already supplied; the inclusion of irrelevant criteria as worthy of attention (colour, shape and texture); the acceptance of irrelevant criteria as evidence of the ability to hypothesize; the use of a set of random objects in which no variables are to be kept constant; and, finally, the choice of a topic for the test which many people, including professional scientists, find baffling and unpredictable. "What are you to do", asked one teacher plaintively, "with a bag of carrots, half of which float and half of which sink?"

There are many other lapses in scientific rigour throughout the test booklets. If these are representative of the level of the "scientific" knowledge, understanding and thinking of those who write the tests, what hope is there for the teachers and the pupils who are subject to the statutory requirements of the Education Act? □

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Doubt afflicts Swiss anniversary

John Maddox

This month's celebration of 700 years of the Swiss Confederation is tinged with anxiety about relationships with Europe, the poor recruitment of students to science and engineering and the problems of cosmopolitanism.

SWITZERLAND seems to be moving towards the celebration of the Swiss Confederation's 700th anniversary with the enthusiasm of a busy person bidden to celebrate the birthday of an ageing aunt. The occasion will of course be a public holiday, there will be some great parties in the mountain villages while great orchestras from all over Europe will entertain the bourgeois populations of Geneva, Lausanne and Zurich. In due course, the implied virtue of having lasted the best part of a millennium will make people feel good — or even better than they do already.

The sense of well-being is now legendary, but it is easily overlooked that its economic component is largely a phenomenon of the years since the Second World War. Switzerland shared, for example, in the Great Depression of the 1930s, as did the rest of Europe. But post-war economic growth, sustained by monetary policies consonant with Swiss traditions of thrift and by banks that are not nearly as conservative as they are made out to be, has generated economic growth within Switzerland and, perhaps more important, the export of capital through multinational companies and other channels.

The less tangible components of well-being are what this month's celebration should be about. Swiss historians are embarrassed by the way in which Schiller and others embellished the legend of

William Tell, but the confederation does owe its existence to the compact reached in 1291 between people around Lake Lucerne to resist rule by local and regional feudal lords. (Other cantons have accreted to the confederation over the years, some only in the nineteenth century, but the latest entrant — Jura — was formed only in 1978, by the fission of a French-speaking enclave from the canton of Berne.).

To the extent that the origin of the confederation was self-defence, the present tradition of well-armed neutrality is logical enough. Similarly, Swiss respect for Switzerland's own laws follows the tradition of self-government which was a necessary consequence of the independence won in 1291. Libertarian influences have been further reinforced by the part played by Switzerland in the reformation of the sixteenth century. Put a well ordered prosperous country such as this in a landscape as varied and breathtaking as that of Switzerland, and the explanation of its remarkable contentment is not far to seek.

Meanwhile, by the bad luck which partly explains why the 700th anniversary will be a low-key affair, Switzerland is also preoccupied with contemporary problems more urgent even than the obvious question of whether the confederation will last another century.

Three issues stand out. Should Switzerland join the European Communities,

and can it survive either if it does or does not? Can Switzerland remain the prosperous place it is if it continues to generate a relatively small portion of the technical skill on which it depends from its own human resources? And can a place so ostentatiously cosmopolitan avoid becoming Europe's melting-pot? The three questions are, of course, different versions of each other.

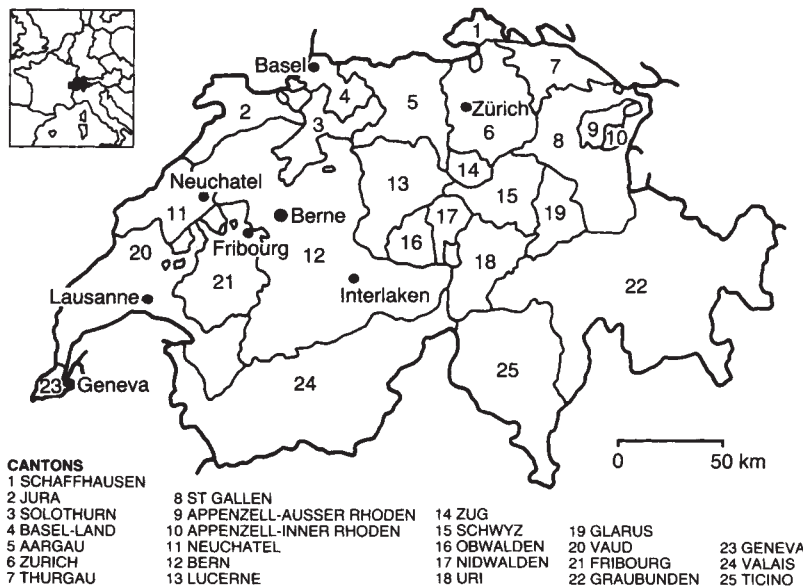
What follows is an account of how a few handfuls of Swiss and Swiss-based scientists regard this amalgam of problems, gathered on a journey between Geneva (the English name for *Genève* or *Genf*) and Basle (as *Bâle* or *Basel* are translated into English from the French and German respectively).

Community membership

Switzerland's future relationship with the European Communities (EC) is a vividly live issue, and for several reasons. The common anxiety is that Switzerland may be left out, and thus put at an economic disadvantage relative to its neighbours. (Switzerland, with neighbouring Austria, is already a member of the predominantly Scandinavian European Free Trade Association, which is in the thick of negotiations with Brussels over a closer relationship, but the governments of both Austria and Sweden have declared their interest in full membership.)

It is natural that the large technically based multinational companies such as Nestlé and Ciba-Geigy should be enthusiastic supporters of Swiss membership of the EC, even though, with manufacturing plants and laboratories scattered across Europe and North America, they are unlikely to be disadvantaged by the single market due to become a reality from the beginning of 1993. Smaller Swiss enterprises, especially those that export large proportions of their product, are much more at risk, and are doing what they can to protect themselves by forming alliances with companies inside the community.

Generally, French-speaking Switzerland is more enthusiastic for membership than the German-speaking cantons to the north. Interestingly, the federal government is also mildly encouraging of membership. Government officials in Berne have been arguing for some years that Switzerland already makes a substantial contribution to the well-being of Europe as a whole, notably by the large numbers



Divided loyalties the determination of the cantons to keep their autonomy means that a 'federal' solution to running the universities is fiercely resisted.

of students from elsewhere (typically more than 15 per cent) at Swiss universities and other educational institutions. Now the government appears to be seeking to influence opinion in general by emphasizing the potential contributions that Switzerland might make to the rest of Europe in other fields as well.

Yet Switzerland, as sophisticated as it is rich, abounds with opinions on the European Communities which are surprising for their naivete. A standard view is that "Switzerland has no choice but to join, but could not accept the conditions that go with membership". The most curious feature of this opinion is that it appears not to acknowledge that even the most draconian-seeming community directives derive from the Treaty of Rome and the requirement that the European Commission should make a level economic playing-field.

Two features of the community stick in Swiss throats; the fear that the habit of EC members of telling each other what laws to adopt would be incompatible with the Swiss tradition of direct democracy, in which all important and many unimportant issues are decided by national referendum. (There is also a particularly fractious issue, already joined between Switzerland and the EC, over the community's specification of the weight and size of trucks that may travel on European roads.) And there is particular anxiety, embedded in ignorance, over the requirements of the EC that citizens of its member states can work and live where they choose.

The last point is paradoxically perplexing to the Swiss, whose cosmopolitanism is both renowned and economically necessary. (In cantons such as Geneva, for example, some 30 per cent of the residents are non-Swiss. In the country as a whole, roughly a fifth of the labour force comes from elsewhere.) Despite the dependence of these 6.5 million energetic people on workers from elsewhere, residence is strictly regulated at the canton level; well-placed employers can win work permits for the people they need, but only after demonstrating (much as in the United States) that there are no Swiss who could do the jobs they seek to fill. And the right to live in Switzerland lasts only for the duration of working life (but university professors and some others are privileged).

The familiarity of this practice no doubt explains why even well informed Swiss are often astonished that the European Communities allow people literally to live in whichever member state they choose. One Swiss enthusiast for membership remarked casually that "of course, we'd have to get that changed".

At the other end of the spectrum are the opinions of those who hold that

Switzerland has been made complacent by several decades of prosperity. "People are too comfortable", says Dr Max Wilhelm, head of research and development at Ciba-Geigy's pharmaceutical division at Basel. He argues that the pleasures of the Swiss way of life have robbed young men and women of energy and drive, and that the tradition of military neutrality has encouraged them to hide from competition, which may in the long run rob Switzerland of its capacity for innovation. "I may lose my Swiss passport for saying this", but they need a shake-up such as membership of the EC would bring.

One alarming consequence of the general sense of comfort is the apparently growing difficulty of persuading Swiss employees of the corporation to work for a stint at Ciba-Geigy's plants and laboratories overseas. The old rule that those appointed to the company's divisional management committees should have spent a substantial spell outside Switzerland is no longer always followed. (But Ciba-Geigy — like many other Swiss multinationals — has now dramatically broken with the other tradition that its most senior directors should be Swiss — "even a few years ago, that wouldn't have happened", says Wilhelm.)

The only certainty about the question whether Switzerland should join the European Communities is that the decision will be slow, perhaps painfully so. There are some who say that they cannot understand how the country could make the transition to membership without a constitutional change in advance that would delegate more power to the federal government. Others take the view that a snail's pace will be best, allowing Switzerland to judge whether 1993 proves to be significant before making such a decision. But, to the extent that opinions appear already to be divided geographically, the issue of EC membership could be more seriously divisive even than the campaign to create the new Canton of Jura in 1978.

Universities

Whatever happens on this huge question, many other prospective changes could change the way that Switzerland feels. One of the more immediate and important issues is that of the universities and their management which, like any Swiss institutions, are fond of emphasizing how the way in which they now conduct themselves has been shaped by decisions taken in the distant past.

Swiss universities are not federal institutions, but are financed and managed by individual cantons. The arrangement has some virtues — notably that universities can hope to command the loyalty and the pride of local politicians — but there are also disadvantages now often painfully apparent. One academic says that the

most serious consequence is that, within the structure of his university (Geneva), "nobody, not the rector and not the dean and not even the head of a department, has power". And the consequence of that is that administration is needlessly cumbersome and inflexible.

This explains why, at many of the seven universities, there are departments staffed on a scale for teaching many more students than there are. This is a splendid luxury for the academics concerned, who thus have more time for research. But whether the arrangement makes national sense is another matter. Industry researchers also complain that a country the size of Switzerland does not need seven departments of chemistry or schools of pharmacy. But canton governments seem as jealous of their autonomy now as they were in 1291 (and many of them spend a third of their annual budget on education at all levels); especially because making the university system federal would necessarily be a prelude to rationalization, the prospects for change are not bright.

There is also a fair amount of cantonal bumbledom, it appears. Although Swiss universities are as vigorous as any industrial company in their pursuit of enlightened cosmopolitanism, in the eyes of the canton government their candidates may have only a low priority (compared with, say, tax-paying corporations) in the award of labour permits.

In much the same spirit, the present arrangements inhibit what many university academics consider to be the necessary concentration of resources on outstanding groups or departments. Switzerland has what the British would call a 'dual-support' system for research, with often generous support (which may be negotiated directly between an academic and the cantonal government) from the canton and the smallish grants (by international standards) provided competitively by the national research council. Two academics agreed the other day with an industrial research director that, in these circumstances, none of them could hope to compete effectively with their colleagues at "ETH".

That, of course, is the federal polytechnic at Zurich, (the Eidgenössische Technische Hochschule) which, like its younger cousin at Lausanne, is directly financed by the government in Berne. There is no law of nature that requires directly supported federal institutions always to be superior, and indeed the Lausanne Polytechnic is still making its reputation (in fields as different as plasma physics and automation), but ETH enjoys much of the credit for the achievements of Swiss engineering this century (not to mention Einstein's first degree). It is no surprise that it should be an outstanding institution.

But how can two technical universities and seven general universities (mostly dominated by arts, the humanities and law) supply Switzerland's need of skill? Both academics and industrial researchers are worried about the recruitment of young people into science, engineering and mathematics. There are the usual explanations: jobs in banking and public administration offer better salaries, young Swiss have been turned away from science by alarms about the environment, and so on.

So should not academics do something to explain that science is still interesting, and what might they do? One academic explained the other day that he had been invited to contribute to the exhibition mounted this year at Zurich, but that in the end he had not done so because of examinations and other diversions, and that now he felt slightly guilty after reading the poor review of the exhibit in *Nature* (20 June, 351, 597; 1991).

Cosmopolitanism

But the plain truth is that Swiss organizations have come to rely on the rest of the world for much of their technical skill. The director of the Glaxo Institute of Molecular Biology at Geneva, Dr Jonathan Knowles, boasts that there are 22 nationalities represented in his staff of 135 (many of whom have been inherited from the Geneva arm of the biotechnology company Biogen before that retreated to the United States). Elsewhere, at Ciba-Geigy, for example, it seems openly to be recognized that countries elsewhere are often sources of skills that Switzerland does not supply. The company deliberately recruits internationally, but says that, for example, Britain is the best source of research-minded physicians (able to conduct clinical trials) and of high-research pharmacologists.

What is Switzerland like as a research base? Three of the institutions between Geneva and Basle (the Glaxo Institute and the Battelle Institute at Geneva and the International Institute for Management Development at Lausanne) need not be in Switzerland at all, but might as well be sited elsewhere in Europe, where costs might also be reduced.

Knowles is disarmingly open about the Glaxo institute, which has just moved from a featureless laboratory block on the Battelle site to a splendidly light and airy laboratory built within what was an empty shell of a modern building only last year. He says that he does not know whether, if Glaxo had not bought the Swiss rump of Biogen it would have chosen Switzerland as the site for a basic research institute. But, given the historical accident, there could hardly be a better place. "Nowhere else", Knowles says by way of illustration, would it be possible to design a new laboratory, with

complicated air-cleaning equipment threaded along every ceiling, and then see the work completed exactly at the prescribed time, and at a cost "within a penny" of the estimate.

It also helps that Switzerland has a policy on workers from elsewhere, even if the policy sometimes appears irksome and restrictive. People embarking on recruitment, for example, will usually know where they stand on labour permits in advance. Laboratories that could as well be sited elsewhere seem to be especially well placed; the mere thought that they might be up and off at any time seems powerfully to influence cantonal officials.

Battelle's location at Geneva is differently explained. When Battelle at Columbus, Ohio, decided in 1952 to take contract research to Europe in the wake of the Marshall Plan, it had hoped to found one laboratory in France and one in Germany. But that happened to be a time when France and Germany were at loggerheads with the result that there is now a Battelle Institute at Frankfurt and another in French-speaking Switzerland.

Somewhat ruefully, Mr Roland Adoutte, the head of the contracts division at Geneva's Battelle, points out that there is one sense in which the location could hardly be worse. In Switzerland (again like Japan), government support for industrial research is negligible, with the result that what government support there is for Battelle's work comes from elsewhere in Europe.

Adoutte says that Switzerland nevertheless provided an environment in which the institute was able to pioneer a novel way of supporting contract research in which the institute, instead of waiting for clients to come knocking on the door, will itself finance innovative research with the objective, then, of interesting manufacturers in particular applications of the new development. In such a case, the institute will own the intellectual property. Proactive research is one name for the arrangement, but "speculative research" might be a better one.

As it happens, the organization has recently decided to develop on the Geneva site (which has shrunk a little in the past few years) a new biotechnology division, until recently located at Frankfurt. The intention seems to be that the new division will earn some bread and butter by providing more or less routine testing services, but there will be speculative research as well.

IMD (the International Institute for Management Development) is similarly at Lausanne for historical reasons; it occupies the site on which Nestlé founded an in-house management school after the Second World War, and which has now merged with another management school

to form what Dr Morgan Gould, the associate director of research, claims is an academic institution on a par with Europe's other centre of management research and training at Fontainebleau, outside Paris.

Again, the intention is to recruit people to the faculty internationally. Again, there appears to be little difficulty. Geographical centrality helps, as does access and the availability of accommodation (for people following the year-long MBA course).

So are the Swiss good managers, as their reputations would suggest? People are a little diffident about answering, but IMD has carried out some studies of the decline, fall and partial reconstitution of the Swiss watch industry, which makes plain for future management students to see that electronic watches were indeed first developed in Switzerland, not Japan. But the failure to recognize that technically superior products might also be manufactured cheaply was a management failure of some substance.

Much in the same spirit of showing that not everything that the Swiss touch promptly turns to gold, there is the example of the electronics industry, in which Switzerland seemed on the way to excellence until about 1960, but which is not now the economy's strongest suit.

But food has done well by the Swiss, both in the form of traditional farmhouse cheeses and as grist for the mill of Nestlé, one of the largest companies in the world (with a turnover approaching SF50,000 million a year).

On the research side, which must be among the world's most colourful — more than 60 chefs are employed on trying out recipes of various kinds — there is a total of 2,600 scientists and other professionally qualified people, drawn from more than 50 disciplines. Just over 1,000 of these are based in Switzerland (many of them at a new research centre above Lausanne). Again the policy is cosmopolitan.

For what it is worth, the Institute of Food Technology (part of ETH) appears to be a source of many able people, but specialists of other kinds — veterinarians, for example — must be found elsewhere. Of necessity, the work is strictly practical and even business orientated (but there is a programme of nutrition research of which many academic institutions would be proud). Yet questions such as how to ensure that a cup of instant coffee will be filled with the aroma of the original beans attract the most solemn attention, as does the disposal of unwanted debris from coffee beans. (One scheme is to compost them.)

Sheer size is Nestlé's outstanding attribute, but Switzerland's reputation for industry rests to a large extent on the pharmaceutical industry and its parent,

the heavy chemicals industry, centred on Basle on the Rhine.

It is a remarkable concentration of industry and research in what is almost a city-centre site. From the top of one of the Ciba-Geigy research blocks, one can see the old Geigy administration building and the production plants beyond that. In the other direction across the city, perhaps 6 or 7 kilometres away, there are buildings occupied by Hoffman-La Roche. Sandoz is around the corner.

Pharmaceuticals account for more than a third of the Ciba-Geigy business, worth some SF6,000 million in sales each year. The strategy, as explained by Wilhelm, is ostentatiously multinational. There is no choice for a pharmaceutical company seeking to make the most of the occasional discoveries of new drugs than to maintain research and development competence in its chief markets so as to satisfy the local regulatory agencies. With 40 per cent of Ciba-Geigy's sales in the United States, it is natural that half of its development effort and a third of its research should also be there.

Basle, however, has hung onto the lion's share of the research, perhaps 60 per cent of it. (Roughly 30 per cent of total research and development is research, Wilhelm estimates, and the total annual cost of both activities is SF900 million.) Roughly a third of the 1,300 research and development people on the Basle site are non-Swiss, but the proportion of other nationalities among the newly hired has risen to 70 per cent — a striking sign of the acute shortage of indigenous skill.

There may soon be many more researchers. Ciba-Geigy's pharmaceutical division seems to have hit a good patch, with several new drugs near the market. Among other things, it is planning a new life sciences division, chiefly concerned with disease mechanisms. But, like many other companies of its kind, Ciba-Geigy remains unrepentantly cool about biotechnology as a direct route to new medicines.

Wilhelm says it has always been his line that "biotechnology is just another technology" (but he reckons that there are 200 people on the Basle site who are skilled practitioners in the field). And while acknowledging that biotechnology is a way of making molecules that otherwise occur only naturally, he insists that "I prefer small molecules" as drugs.

The reasons? The products of biotechnology are rarely pure in the degree now required by the regulatory authorities, and often unspecific (and so inherently liable to side effects). Of necessity, they do not make oral medicines, while the drug delivery problem is especially perplexing, he says: the body generates vital protein molecules in the tissues in which they are needed, which is a far cry

from loading the general circulation with them.

What is there on the horizon? Ciba-Geigy plans to continue to concentrate on the fields in which it is already strong — drugs for the central nervous system (epilepsy, for example), cardiovascular drugs, medicines for bone diseases (including rheumatoid arthritis as well as osteoporosis) and the search for antitumour drugs. But the company has recently taken an interest in chimaeric antibodies, a search for both drugs and vaccines for the treatment and prophylaxis of AIDS and an exploration of the IgE component of the immune system in the causation of allergic diseases.

On the face of things, these preoccupations closely resemble those of other companies active in the search for drugs. It is interesting, for example, that the Glaxo Institute of Geneva has developed the chick retina as a model of the mechanism of cell death in the central nervous system, believing that it may thus be able to throw some light on the phenomenon that more generally characterizes the ageing process (and perhaps even Alzheimer's disease). And Battelle, ever practical, has taken to offering an off-the-shelf service to pharmaceutical companies that, for a predetermined price, will assay a given compound for its affinity with up to forty recently characterized cell-membrane receptors, many of them neuronal.

Green Switzerland?

Told this way, the Swiss pharmaceutical industry seems well-set to be as much a guarantor of Swiss prosperity in the future as in the past. But there is one cloud on the horizon — the risk that Switzerland may become restrictively "green". Wilhelm, a courteous man with impeccable manners, thumps the table with the heel of his palm when he contemplates the prospect.

It is a curious business. Switzerland, of course, has a long-standing tradition of regard for its own landscape, as well as an innate tidiness in its management of the built environment. But in the past three years, there has been a powerful movement to extend the restriction of offences to the eye and other senses by legislative restrictions on research.

The case of the new restrictions on the use of animals in research, for example, is causing considerable anxiety. A nationwide referendum that would have restricted the use of animals to procedures with direct medical benefit failed to win the necessary majority, but the federal government has sought to meet the organizers of the referendum half-way by legislation that requires that laboratory procedures should not cause pain to animals (a common feature of other restructions on the use of animals in

research) and by the creation of a veterinary inspectorate that must approve in advance all proposals for the use of animals in the laboratory.

Complaints from researchers have centred not on the criteria of what is allowed, but on the delay in winning approval.

But there is more. Both human embryo research and genetic manipulation are likely to recur on the list of referendum issues.

There seems to be a general understanding that research with human embryos will not be allowed, the inevitable over-production of embryos in *in vitro* fertilization notwithstanding.

To the relief of many people in research, a debate in the federal parliament earlier this year established in the minds of politicians and the general public the distinction between genetic manipulation as a laboratory procedure and that which results in the production of viable and novel organisms. However, the status of the latter still remains to be determined.

An important part of what seems to be a mounting difficulty is that many green ordinances are promulgated and enforced at the level of canton governments, which means that the practical consequences can vary markedly from one part of this small country to another.

Wilhelm, at Ciba-Geigy, believes there is a strong movement in the region "against what industry is doing". He is especially incensed that the smallest industrial accident will be blown up by the local newspapers with sensationalist headlines such as "FIRE AT CIBA-GEIGY!". Four years, he says, have been wasted on negotiations to build an incinerator for the company's toxic waste, but the issue may eventually be settled only in the courts.

In reality, it is probably not accidental that these issues are strongest in the part of Switzerland most sceptical of membership of the European Communities. Making Switzerland different, and more restrictive than other European countries, may also help to keep it separate. At this stage, moreover, nobody is suggesting that the restrictions are so onerous that the great corporations would pack their bags and move elsewhere.

That is yet another reason why Switzerland is unlikely to be able to reach a quick decision about its relationship with its neighbour states (all of whom, with the exception of Austria, belong to the European Communities). The worry is that the issue is so important that it could also become divisive. If the European Communities become as prosperous as they hope, some cantons may think of seeking membership on their own account. But that, in the Swiss tradition, will take decades. □

Is Big Brother really watching us?

Is it worse to have supplied Iraq with materials from which poison gas can be made, or to have supplied without knowing the fate of the material? That question should now torment the British government.

MODERN governments cultivate the fancy that they are all-seeing and all-knowing, which is natural enough. If their reputations were otherwise, they would quickly find that individuals with whom they are thrust into relationships on matters as different as the payment of taxes and the regulation of prescription drugs would fail to take them seriously. But the British government, in an accident-prone patch for several weeks, has run into a nasty bout of trouble over export licences for equipment destined for Iraq.

Among other things, British companies have been substantial suppliers of chemicals that could have been (and probably were) used for the manufacture of chemical munitions. But these exports, far from being clandestine, were open and above board, duly licensed by the Department of Trade and Industry and recorded in a list of exports to Iraq supplied to the Trade and Industry Committee of the House of Commons. And that committee (which published the list in an annexe to its report), which was bent on getting to the bottom of the supply of parts for Iraq's supergun by British companies, appears to have been no more aware of the significance of what had happened than the British government itself.

The immediate and political importance of these events cannot easily be foretold. Mr Peter Lilley, the Secretary of State for Trade and Industry, has promised to give fuller details of what really happened when the British Parliament reassembles in the second half of October. Forgetfulness (or a general election) may make that ordeal unnecessary. That is why the significance of what has now emerged must be differently judged. It is both an illustration of how governments function (or can fail to function) and a measure of how much weight can be carried by arrangements to control the spread of dangerous weapons by means of agreements among potential suppliers that they will not supply.

The best-known of these is what is called the London Club agreement — drawn up in September 1977 between the chief suppliers of nuclear equipment and fuel, not to supply to customers suspected of making nuclear explosives. At the time, there was great rejoicing that both the Soviet Union and France (not a signatory of the Nuclear Nonproliferation Treaty) had signed up. But it is also

relevant that in the past few months, since the end of the war in the Persian Gulf, there has been strong support for a Japanese proposal for an international register of supplies of conventional arms to the world's potential trouble-makers.

The legislative history of this business is not simple. The British government can require British companies and persons to apply for export licences for sensitive equipment under the Import, Export and Customs Powers (Defence) Act 1939, which goes back to the outbreak of the Second World War, and which is kept up to date by means of statutory orders (which even Members of Parliament do not have time to read).

The law obliges those wishing to export sensitive equipment to apply for a licence from the Department of Trade and Industry. To fail to appreciate that a licence may be necessary is a criminal offence; two people engaged in supplying parts for Iraq's supergun were indeed thus charged last year.

One elementary lesson from the past few days' fracas is that the British government needs modern legislation to do the job it has set itself, even if the "procedures are well understood by those in export business". As things are, those wishing to export sensitive military equipment to governments elsewhere must often be tempted not to apply for the licences legally required; the chance of being caught is small, the chance of being prosecuted successfully even if caught can be no greater.

The evidence of the list of material and equipment authorized for shipment to Iraq from Britain in the past three years can only have assured exporters that the Department of Trade and Industry was, in any case, mostly on their side. Among the bulk chemicals sent to Iraq were £154,000-worth of thiodiglycol in 1988 and £33,000-worth the following year — perhaps 100 tonnes in total. It is true, of course, that thiodiglycol is a wetting agent, and is used in making sure that ink in a ball-point pen will smoothly cover the surface of the writing head. But at a milligram per pen, that would be enough to produce 100,000 million ball-point pens. Otherwise, there would be few other uses for the material except to convert it into mustard gas.

Similar conclusions will be reached about the substantial amounts of organic

phosphonates and phosphonites sold to Iraq over the same period; they are convenient starting points in the manufacture of nerve gases such as Sarin (as well as, of course, of pesticides). And then there is the curious revelation that quantities of plutonium, depleted uranium and zirconium were exported.

These are so distinctively nuclear materials that Mr Lilley has thought it prudent to break his trappist vow — "no more details until October" — by emphasizing that only gram quantities of these materials — "far too little to make a bomb" — were ever shipped, presumably for use in medicine. But suppose, say in 1989, that Iraq had been embarked on a nuclear programme on the scale now revealed by four successive inspections of its facilities, and that it was then apparent that two or three years would have to pass before indigenous plutonium would be available. Would it not be handy, to say the least, to be able to buy a little of the eventual product on the market so as to check all those figures of cross-sections published in the text-books?

So how could such a mess have arisen? Mercifully, the memorandum now published tells all. Each year, the group of 20 people dealing with export licences of sensitive equipment from Britain have to process some 70,000 applications — say ten per person per day. From their base in the Department of Trade and Industry, they have to consult counterparts at the Foreign Office, the Ministry of Defence and less well advertized branches of the government, and well as deal with telephone enquiries pleading that without an instant licence, valuable export trade will be lost. Archlessly, the memorandum says that most of the efforts of this group have been directed at monitoring exports to the Soviet Union, eastern Europe and China. By default, Iraq seems to have enjoyed over the years a steady supply of night-vision equipment, and computer hardware and navigation equipment of unspecified function and design.

Several issues of principle arise. Jobs like these, if they are to be done well, should be done internationally, so that people are not looking over their shoulders at the balance of payments. The law should be explicit and its administration transparent. And the people who do the work should be imaginative, able occasionally to put two and two together to make five. **John Maddox**

Passive primate protection

John P. Moore and Robin A. Weiss

ON page 434 of this issue, Ward *et al.*¹ demonstrate that a soluble, recombinant immunoglobulin chimaera of CD4 (sCD4-IgG) prevents infection of chimpanzees by the IIIB strain of human immunodeficiency virus type 1. In conceptually similar experiments, Putkonen *et al.*² (page 436) show that passive immunization of rhesus macaques with serum from vaccinated, immune macaques protects the animals from subsequent challenge with HIV-2 or simian immunodeficiency virus (SIV). The results of these passive protection experiments are important: those of Ward *et al.* imply that HIV infection can be blocked pharmacologically under certain circumstances *in vivo*, and the serum protection experiment adds to the present optimism about the eventual production of an effective vaccine against HIV in humans.

Not unreasonably, both experiments were designed to maximize the chances of successful protection in that live virus challenge was initiated intravenously when serum sCD4-IgG or Ig levels were near their highest. The degree of tolerance in timing and dose of sCD4-IgG or Ig remains to be evaluated, but protection from HIV infection under field conditions is likely to be much more difficult³. As Ward *et al.* point out, a promising feature of sCD4-IgG is its transfer across the placenta, which raises the hope that an appropriate dosage regimen may protect against maternal-fetal HIV infection. The kinetics of this route of transmission are poorly understood, but the world-wide increase in paediatric AIDS warrants serious investigation of any rationally based prophylactic. Yet it is arguable whether sophisticated drugs such as sCD4-IgG will be accessible to those communities where mother-to-child infection is most prevalent. We calculate that the chimpanzees received 6–9 g of sCD4-IgG, an expensive recombinant protein.

Molecular mimic

The immunoglobulin chimaera⁴ was designed as a molecular mimic of CD4, the principal receptor for HIV. Presumably its mechanism of action *in vivo* resembles that *in vitro*, where it and sCD4 neutralize HIV-1 (IIIB) by competitive inhibition of the virus-cell binding reaction and by irreversibly stripping the envelope glycoprotein gp120 from the virus^{5–7}. Primary isolates of HIV-1 are, however, relatively resistant to neutralization *in vitro* by sCD4 and sCD4-IgG (ref. 8). Our unpublished results (with D. D. Ho and J. A. McKeating) suggest

that, as intact virions, sCD4-resistant primary isolates bind sCD4 and sCD4-IgG with greatly reduced affinity but then retain their gp120 relatively well. Furthermore, Layne *et al.* have shown that extremely high concentrations of receptor blockers such as sCD4-IgG are required as CD4⁺ cell densities approach those found *in vivo*, for example in lymph nodes⁹. Taken together, these factors may limit the efficiency of sCD4 and its derivatives as an antagonist of HIV *in vivo*.

It will be interesting to find out precisely how the passive serum protection demonstrated by Putkonen *et al.* was mediated. Whether the challenge virus was neutralized by antibody or eliminated subsequently by antibody-dependent cytotoxicity is not yet known. Whatever the mechanism, it appears that generation of an appropriate humoral immune response can be protective in macaques. By inference, protective antibodies developed in the donor macaques in response to a killed virus vaccine. This is an important point, as the role of neutralizing antibodies in protection against SIV *in vivo* is somewhat controversial.

In several immunization studies there has been little or no correlation between *in vitro* neutralizing antibody titres and protection^{10–12}. Neither is it clear how neutralizing antibodies work for SIV or HIV-2 *in vitro*. Putkonen *et al.* suggest that neutralization might be mediated through the V3 domain of SIV gp120. But this is not generally accepted to be a potent neutralization epitope for HIV-2/SIV (ref. 13), in marked contrast to HIV-1 where antibodies to the V3 loop have a dominant role in neutralizing virus entry into susceptible cells. In the few successful protection experiments in the HIV-1/chimpanzee model, the V3 loop has been held to be of critical importance^{14,15}. Given the dissimilarity of the principal neutralization epitopes for HIV-1 and HIV-2/SIV, and differences in cell tropism for these viruses operating at the level of virus entry¹⁶, it is a worrisome possibility that experiments on SIV may not be predictive of humoral protection against HIV-1 in humans. Only time will tell, and at present the SIV model is still valid, especially for analysis of the cellular immune response. An important complementary animal model may well be the SCID-Hu mouse, which allows interactions of HIV-1 with a human-like immune system to be evaluated¹⁷.

As our understanding of the interactions between different HIV isolates and

their target cells increases, it is becoming apparent that tissue-culture-adapted isolates of HIV (and SIV) may be inappropriate for use in animal experiments. The commonly used LAV-1/IIIB isolate has not yet induced disease in chimpanzees^{18,19}. But is it now pathogenic for humans? Adaptation to growth in transformed CD4⁺ cells may have attenuated the virulence of LAV-1/IIIB, or otherwise modified the way in which it interacts with susceptible cells *in vivo*. The converse has been demonstrated, in that passage of LAV-1/IIIB in chimpanzees modifies its tropism *in vitro*, although the altered viruses are still not pathogenic in the short-to-medium term *in vivo*^{20,21}.

Dichotomy

A powerful example of the *in vitro* versus *in vivo* dichotomy is the selection for a functionally active SIV *nef* gene *in vivo*, but against it *in vitro*, and the correlation between a functional *nef* gene and SIV pathogenesis²² (see also News and Views²³). Another example is the selection for truncated forms of the SIV transmembrane glycoprotein on passage in human T-cell lines²⁴. HIV-1 isolates that are less well-adapted to cell culture also appear to be apathogenic for chimpanzees^{18,19}, but might be more relevant for mimicking HIV infection of humans.

Now may be the time to put aside HIV and SIV isolates that conveniently grow to high titres in cell lines and to concentrate on isolates closer to those found *in vivo*⁸. "To culture is to disturb"²⁵. For similar reasons, the need for multiple vaccine trials with recombinant forms of LAV-1/IIIB glyco-

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proteins from different manufacturers should be re-evaluated. Because the divergent V3 loop of the LAV-1/IIIB isolate may yield atypical neutralization profiles, comparative trials with more typical isolates might be of greater value. The politics of *env* are real, but let us hope that the ability

of scientists to change is not inversely proportional to that of our opponent, HIV. □

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MOLECULAR ELECTRONICS

Bringing molecules to order

Richard Friend

METHODS that allow controlled growth of ordered films of molecular or polymeric materials are much sought after for a range of potential applications. With this in mind, Wittmann and Smith take a fresh look, on page 414 of this issue¹, at experiments performed some years ago on the nature of friction processes when poly(tetrafluoroethylene) is drawn across a smooth surface². The friction produces highly ordered and oriented thin layers of poly(tetrafluoroethylene) which Wittmann and Smith now find can be used as versatile and effective substrates for the growth of ordered films of molecular materials. This provides an important new method for preparing a wide range of structures and will be of particular interest to those working on the development of molecular materials for electronics, or molecular electronics.

Poly(tetrafluoroethylene) is well known to show very low coefficients of friction against a range of surfaces, and is widely used for this property. Pooley and Tabor³ studied the processes that occur as this polymer is drawn across a smooth surface, such as glass, and found that a thin (typically 5 nm) film of the polymer is formed on the surface as polymer chains that are in contact with the surface are drawn out of the bulk. Slipping occurs not at the interface, but between the bulk of the polymer and this thin surface layer which is highly oriented. The low friction arises as aligned chains slide freely past one another. It is this thin layer, transferred to the underlying surface, that is used by Wittmann and Smith as a substrate on which oriented films can be grown.

For molecular materials which have a strong tendency to order, such as liquid crystals, oriented substrates are widely used to bring about alignment of molecular layers in a well-defined direction on a substrate (rubbed polyimide layers are used in most liquid crystal displays). The alignment of a nematic liquid crystal on thin, oriented poly(tetrafluoroethylene) layers produced frictionally has previously been used to provide an image of the underlying order in the polymer layer under a

polarizing microscope⁴. Wittmann and Smith have shown that a much wider range of materials can be oriented in this way, including many, such as polyaniline, that do not show a strong tendency to order.

Control of order in polymers is central to the control of both mechanical and electronic properties. A well-known example is the range of mechanical properties which can be obtained in polyethylene of varying molecular weight and draw ratio. As a low-molecular-weight material, its elastic moduli and strength are not impressive, but highly oriented ultrahigh-molecular-weight polyethylene shows a modulus and tensile strength close to the theoretical strength determined by the intrachain covalent carbon-carbon bond strength⁵.

Conductivity

Turning to electronic properties, conjugated polymers such as polyacetylene conduct metallically when oxidized or reduced, and levels of conductivity have risen to become comparable to those of good metals (copper's is 600,000 siemens (S) per centimetre) since interest first arose in this in the late 1970s. What has been less clear is the way in which conductivity is controlled by order in the polymer. But recent work on drawn fibres of various conjugated polymers reveals a linear relationship between electrical conductivity on the one hand and tensile strength or Young's modulus on the other, rising with increasing draw ratio. Values of 30,000 S cm⁻¹ and 0.9 or 50 gigapascals, respectively, are reached for polyacetylene⁶ and 1,200 S cm⁻¹ and 0.7 or 35 gigapascals for poly(2,5-dimethoxy phenylene vinylene) (ref. 7). This correlation between modulus and conductivity provides an important clue to the processes that limit the electronic mobility. The high levels of both that are reached for high draw ratios point to many areas of application.

Molecular materials are harder to process than polymers, but there is interest in electronic properties ranging from nonlinear optical properties, such as second-harmonic generation, to metallic

and superconducting behaviour. Growth, layer by layer, of films by the Langmuir-Blodgett technique is well developed, but this is constrained by the requirement that the material to be deposited forms a Langmuir film on the surface of the transfer medium (usually water). Most materials put down in this way are designed with long aliphatic tails which cause the molecules to stand on end on the water surface. It can be undesirable, however, to have a large volume fraction of the film that is electronically inactive, and there are now some rigid-rod polymers (phthalocyanato-polysiloxanes⁸ and polyglutamates⁹) which have been developed to lie flat on the water surface. When transferred to form the Langmuir-Blodgett film these align with the direction of draw of the substrate, and provide films of high quality.

Electronic mobilities in organic semiconductors are strongly influenced by the level of order in the material, and these have usually been found to be extremely low, of the order of 10⁻⁴ cm² V⁻¹ s⁻¹ at room temperature, and characteristic of transport by thermally-activated motion from one molecular site to the next. Garnier *et al.*^{10,11} have shown that it is possible to use sexithiophene (an oligomer of the conjugated polymer polythiophene) as the active layer in a field effect transistor and to achieve mobilities as high as 0.4 cm² V⁻¹ s⁻¹. These molecular films were deposited by the simple means of sublimation *in vacuo*, but it now seems that these structures may be unexpectedly well ordered. Recent X-ray measurements on similar films of thiophene oligomers show a highly-ordered, crystalline packing of molecules giving good intermolecular contact¹². The technique now developed by Wittmann and Smith provides a particularly versatile method that will allow fabrication of ordered molecular films drawn from a wider range of materials than previously possible. □

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More Hubble trouble?

John Peacock

How old is the Universe and how far are the furthest objects we can see? These are questions addressed by astronomers trying to determine the Hubble constant, the measure of the rate at which the Universe is expanding. Since the early 1960s, observers have been unable to agree where in the range $50\text{--}100\text{ km s}^{-1}\text{ Mpc}^{-1}$ the Hubble constant lies. But the appearance of two recent surveys^{1,2} in remarkable agreement with one another suggests the field may be narrowing drastically, perhaps with uncomfortable consequences.

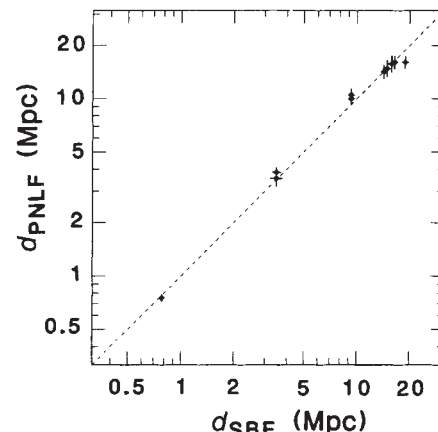
The failure to pin down the extragalactic distance scale has long been a source of despair and cynicism for cosmologists, who are consumers of the efforts to measure the Hubble constant (H_0). The long-running battle between two schools of H_0 values that differ by a factor of two has led to the parameter h ($=H_0/100\text{ km s}^{-1}\text{ Mpc}^{-1}$) being the most overworked symbol in modern cosmology papers. For many purposes the uncertainty in h does not matter: researchers quickly learn to use the standard, if inelegant, distance unit of $h^{-1}\text{ Mpc}$, which is directly observable (distance $= 3,000 \times$ redshift in these units, at least for nearby objects with small redshifts). However, awkward fundamental questions relating to the distance scale cannot always be shuffled away in this manner.

The main skeleton in the closet of cosmology is the age of the Universe: this is $10h^{-1}\text{ Gyr}$ ($1\text{ Gyr} = 10^9\text{ yr}$) for a constant expansion, but only $2/3$ of this if the Universe has the critical density ($\Omega = 1$) which eventually halts the expansion and which is preferred by theorists and is now supported by some experimental data. Given that nuclear cosmochronology³ puts a firm lower limit to the age at 10 Gyr , and that dating of stars in globular clusters⁴ has long favoured ages in excess of $12\text{--}14\text{ Gyr}$, most disinterested cosmologists have preferred to assume that h will eventually be shown to lie closer to 0.5 than to 1 . However, cosmologists may soon lose the freedom to choose whatever value of h they find convenient.

One of the (potentially) cleanest methods for measuring h comes from gravitational lensing. The light from distant quasars can be gravitationally focused by foreground galaxies to give multiple images of a single object. The difference in light travel times for the paths corresponding to the different images scales as h^{-1} . Last month, Roberts *et al.*⁵ presented good evidence that this delay has now been measured to be around 510 days for the double quasar

0957+561, the first gravitational lens to be found. (Their figure is about 25 per cent higher than those previously put forward by optical astronomers, a discrepancy which needs to be understood.)

Converting this delay into a measurement of h requires a model for the lensing galaxy; the simplest one which can



A comparative plot of galaxy distances derived from the planetary nebula luminosity function (PNLF) compared with those deduced from surface brightness fluctuations (SBF). Given the long history of controversy in the extragalactic distance scale, the agreement is astonishingly good. (From ref. 2.)

explain the observed image structure has two components: a galaxy with an isothermal halo with velocity dispersion σ_v , and a uniform screen of cluster dark matter. The two combine in some unknown proportion to produce the observed image splitting, and the time delay is proportional to σ_v^2/h — that is, a greater path difference is induced in those models for which the total deflecting mass is more centrally condensed. Rhee⁶ has measured σ_v , and so h can in principle be deduced. The delay recorded by Roberts *et al.* yields low values ($h \approx 0.4\text{--}0.5$), which apparently gives some comfort to those who prefer an old Universe.

But there are pitfalls in the analysis. First, the measured value of σ_v applies to the stars in the central few arcseconds of the lensing galaxy, whereas we need the value for the dark matter at a somewhat larger radius, which is almost certainly higher. Roberts *et al.* introduce a correction factor of $\sqrt{3/2}$, which raises their estimate to $h \approx 0.6\text{--}0.7$; but this correction is really no more than an educated guess. Moreover, further uncertainties are introduced by the distribution of dark matter. Because multiple quasar imaging probes the lens

potential at only a few locations, uncertainties in the lens model will inevitably remain (although these may be reduced by observing distortions in faint background galaxies⁷). At present, the observational uncertainties in σ_v^2 and the time delay limit the precision with which h can be inferred to 30 per cent. Even when these sources of uncertainty are removed, it is not clear that the remaining systematics in modelling the dark matter will be much smaller. In short, although it is impressive that direct distance-scale measurements from gravitational lensing come close to conventional figures, the prospects for obtaining absolute measurements of h accurate to 10 per cent in this way seem rather remote.

Fortunately, the sorry state of the traditional approach to the distance scale, using 'reliable' objects in our Galaxy to build a ladder to remote galaxies, has been transformed in the past year or so by the advent of two new extragalactic distance indicators. The simplest, advocated by Jacoby *et al.*¹, is to use the luminosity function of planetary nebulae. These are clouds of gas ejected by evolving massive stars, and there are both theoretical and empirical grounds for expecting that they will have the same distribution of luminosities in all galaxies, regardless of stellar population. The objects therefore yield a standard candle; distance can be found directly from apparent brightness (magnitude), and a precision of 10 per cent in distance has been claimed.

The other indicator, due to Tonry², is more complicated. Effectively, it is a method for counting the stars in a galaxy, even when the galaxy is too distant for the stars to be resolved. Consider a galaxy which produces a given amount of light: how can we tell whether this is due to a few nearby stars, or to many stars at greater distance? Even though there are very many stars (say N on average in each resolution element), their discrete nature may be detected because Poisson fluctuations will produce $1/\sqrt{N}$ variations in brightness between different pixels. Galaxy images are thus not smooth, but mottled by white noise with a coherence length set by the astronomical resolution; the amplitude of the fluctuations allows one to count the stars, and hence to get relative distances from the observed galaxy magnitudes if the stellar populations are the same in all galaxies.

The trouble is that, even within a single class of galaxy such as ellipticals, the stellar populations do vary. Tonry attempted in the past to calibrate out this variation by using a predicted relation between stellar populations and galaxy colour, which led to a claimed accuracy of 10 per cent for his method.

To have two methods of this accuracy in the field of extragalactic distances already seemed too good to be true, but Tonry has now gone further still. His new data show that, empirically, the colour-based correction is not as expected; using the correct relation reduces the scatter in this method to 5 per cent.

At this point, some scepticism may seem in order. Given either Tonry's or Jacoby's method in isolation, worries about unforeseen systematics would inevitably arise. Remarkably, however, the distances deduced by the two methods are in perfect agreement over a factor 30 in distance from Andromeda (the nearest large galaxy, also termed M31) to the Virgo cluster. This is illustrated in a plot (see figure) that may spell the end of the extragalactic distance controversy. Virgo is far enough away that most of its redshift is due to cosmological expansion, rather than to local gravitational perturbations; moreover, the redshift of Virgo in an ideal smoothly expanding Universe can be estimated quite accurately. The result is that the Hubble constant is determined to within 7 per cent, once the distance, D_{M31} , to Andromeda is known: $h \approx (D_{M31}/0.63 \text{ Mpc})^{-1}$. As the best estimate of D_{M31} (from the brightness of its Cepheid and RR Lyrae stars) is about 0.77 Mpc, this implies $h \approx 0.82$, and an age for the Universe of only 8.1 Gyr, if the density is critical ($\Omega=1$).

It therefore now seems that at least one of the following must be true: there is an error in the distance scale closer than Andromeda; the ages of globular clusters are too high; or the Universe does not have $\Omega = 1$. The second option alone does not help, because nuclear cosmochronology insists on an age above 10 Gyr. The last option is certainly possible; the best we can say is probably $\Omega > 0.3$ in dark matter from studies of peculiar-velocity fields⁸, which requires an age of $8.0h^{-1}$ Gyr. This age would be increased to $9.6h^{-1}$ Gyr if vacuum energy was enlisted to supply the missing $\Omega = 0.7$ to 'close' the Universe. But many cosmologists are wedded to the idea of $\Omega = 1$ in dark matter, and will prefer to focus attention on the local distance scale.

Even pushing the errors on h to their reasonable limits, achieving an age of 13 Gyr in a critical Universe apparently requires $D_{M31} = 1.0 \text{ Mpc}$ — 30 per cent larger than the conventional value. Is this plausible? The standard-candle distance to Andromeda is derived from a comparison with stars in the Magellanic clouds, whose distance requires at least two further steps to tie into local stellar measurements based on parallax due to the Earth's orbit. We may hope for improvements in local distances from the astrometric Hipparcos satellite, which will extend parallax measurements and provide more direct calibration of distances to Cepheid and RR Lyrae stars. In the meantime, however, there has

HELIX FORMATION

Alarums and diversions

Arthur M. Lesk

MEASUREMENTS of α -helix formation in oligopeptides reveal the conformational preferences of individual amino acids. Among the approaches to the quantitative measurement of the relatively strong tendency of alanine for α -helix formation are those described by Kemp *et al.* on page 451 of this issue¹ and by Chakrabarty *et al.* in *Nature* of 13 June². Related work of Kallenbach and coworkers has also appeared recently^{3,4}. These investigations address the question: can the tendency of each amino acid to form an α -helix be expressed in terms of a single parameter, and — if so — what is the value for alanine?

Studies of oligopeptide conformation have a strong claim on the attention of anyone interested in protein structure. The complete amino-acid sequences of proteins determine their three-dimensional conformations, but below the domain level sequence-structure relations are tenuous. However, helical regions from native proteins are implicated as folding intermediates; and conformational preferences are the basis of methods for attempting to predict secondary structure of native proteins and for *de novo* protein design⁵.

Experimental measurements of helix formation are interpreted according to a theoretical model developed originally by Zimm and Bragg⁶. Two thermodynamic parameters characterize the helix-coil transition: σ , which describes the nucleation of a helix, and s , which describes helix propagation. Helix-coil transitions in long polymers are cooperative because propagation is easier than nucleation. With statistical mechanics, it is possible to calculate the helix content as a function of σ , s and the chain length n ; conversely one analyses experimental data to derive values of σ and s . It is the

been an ingenious direct distance measurement for the Magellanic clouds⁹ using Hubble Space Telescope observations of the Supernova 1987A, which gives exactly the conventional result for the distance.

To sum up, it now seems increasingly likely that we live in a Universe with an age of at least 12 Gyr and with $h \geq 0.7$ — numbers which are inconsistent with a matter-dominated model and $\Omega = 1$. Something has to give soon, and it will be fascinating to see whether it is the theorists or the observers who back down first. □

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s values of different amino acids that are interpreted as helix-forming tendencies. The theory can be extended to heteropolymers, using different values of s for residues at different positions in the sequence.

Values of s for the natural amino acids in dilute aqueous salt solutions cannot be determined directly using polypeptides containing only one type of residue, because many of these compounds are insoluble. An early attempt to get round this obstacle was made by Scheraga and coworkers⁷, who used the 'host-guest' method and measured helix-coil transitions in copolymers containing a hydroxypropylglutamine host and one of the natural amino acids. They found that at a temperature of 303 K, σ is about 10^{-4} and s about 1 for all amino acids. Although there is a correlation between the values of s measured this way and conformational preference parameters inferred from statistical analysis of known protein structures, none of the s values for natural amino acids measured by Scheraga *et al.* is larger than 1.2.

The points to emphasize here are that these measurements implied that the helix-preference tendency varies only slightly among natural amino acids, and that the values of σ and s implied that short peptides (degree of polymerization of less than about 100) should not be measurably helical under near-physiological conditions of solvent and temperature.

It was, therefore, a surprise when it emerged that short oligopeptides of natural amino acids — some fewer than 20 acids long — can form α helices in aqueous solution. In some cases the stability arises from electrostatic effects (for example side-chain salt bridges along the helix surface) or dimerization

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Insider dealing

W. KRÄTSCHMER, R. Smalley and their colleagues are getting into buckminsterfullerene like no others. The first group (T. Weiske *et al.* *Angew. Chemie Int. Edn. Engl.* **30**, 884–886; 1991) have invented what they term 'endohedral' chemistry by incorporating helium and neon atoms into the interiors of the carbon shells through high-energy collisions. Pairs of carbon atoms are ablated from the C₆₀ clusters in the collisions, apparently as the inert atoms punch their way in. Smalley and colleagues (T. Guo *et al.* *J. Am. chem. Soc.* **95**, 4948–4950; 1991), not delving so deeply, find it is possible to substitute boron atoms for individual carbon atoms in buckminsterfullerene's shell to produce a Lewis acid that attaches ammonia molecules to its exterior. In the meantime, M. Saunders (*Science* **253**, 330–331; 1991) calculates that the fully hydrogenated fullerane C₆₀H₆₀ is furry on the outside and on the inside, the carbon–carbon bond strain being minimized if several of the hydrogen atoms find their way into the molecule's interior.

Hunting the gene

It has been eight years since the dramatic mapping of the gene for the neurodegenerative disorder Huntington's disease to chromosome 4, yet attempts to assign the gene more accurately to one of two 'candidate' regions near the telomere have been confounded. Concentrating on the more proximal region, G. P. Bates and colleagues have used a combination of genetic and physical mapping methods to show that the distance between two DNA markers thought to flank the Huntington's gene is a mere 2.5 megabases (*Am. J. hum. Genet.* **49**, 7–16; 1991). Although it may still take a considerable time before the gene itself is identified, the signs are encouraging that the boundaries for the search have finally been delineated.

Split decision

New limits on exotic particles, calculated by J. Gratsias, R. J. Scherrer and D. N. Spergel (*Phys. Lett. B* **262**, 298–302; 1991) close off, or at least constrict, a favoured let out for cosmologists whose Big Bang models don't quite predict the observed Universe. The idea is that short-lived, massive particles existing immediately after the Big Bang altered the subsequent evolution of the Universe, but are conveniently no longer around. They are usually disposed of by being made to decay into energetic neutrinos, which barely interact with other material in the Universe. But Gratsias *et al.* show that annihilation of decay neutrinos on cosmic background neutrinos creates photons that would split the light nuclei synthesized in the Big Bang. Solving one problem, it seems, simply creates another.

(packing of hydrophobic faces of amphiphilic helices). Numerous studies of α -helix formation in oligopeptides have been designed to clarify the situation, using variants of these natural sequences, and synthetic sequences designed for systematic variation in amino-acid composition and sequence.

Anyone wishing to participate will have to carry out the following steps. (1) Purify your material; (2) find conditions in which it is at least partially α helical, and prove its structure by circular dichroism or nuclear magnetic resonance (preferably both); (3) measure its fractional helix content in a variety of conditions; (4) verify that the measurements are consistent with the Zimm–Bragg theory; and (5) derive values of σ and s by fitting the data to available equations. Then compare your results with those from earlier work and from the new studies^{1–4}.

The paper by Chakrabarty *et al.*², the latest in a series of reports from Baldwin's group, is a study of an alanine-rich reference peptide and 12 related peptides with Gly substituted for each of the Ala residues. Formation of α helices at 273 K and pH 7 was measured by circular dichroism, and the results fitted to the theory, with the result $\sigma = 0.0029$, $s(\text{Gly}) \sim 0.015$ and $s(\text{Ala})$ is no less than 1.5. As in several other studies, the value for Ala is much higher than that of Scheraga and coworkers⁷.

Kemp and colleagues¹, taking a different approach, have designed an N-terminal 'template' to initiate and 'report' helical structure in an attached oligopeptide. The template, a bridged derivative of the dipeptide Pro-Pro, is a kind of 'flip-flop', in a conformational equilibrium between one state that can form hydrogen bonds to an attached peptide in the α -helical conformation, and another state that cannot form such hydrogen bonds. Thus α -helix formation in the attached peptide will be reflected in a shift in the equilibrium of the template, detectable by NMR. The authors apply this method to alanine-rich oligopeptides at 298 K and pH 1–2. No circular dichroism data are presented, and I must admit that I would be happier with a more rigorous demonstration that no conformations of the attached peptide except the α helix can interact with the template to affect its equilibrium. From the shifts in the equilibrium of the template, the authors infer values of $s(\text{Ala}) \sim 1.0$ and $s(\text{Gly}) \sim 0.3 - 0.6$, consistent with the results of Scheraga and coworkers⁷ but not with those of Chakrabarty *et al.*².

In another application of the host-guest method, Kallenbach and coworkers^{3,4} studied α -helix formation in oligopeptides containing the sequence Glu₄-Lys₄-X₃-Glu₄-Lys₄ in which the

Glu₄Lys₄ blocks provided solubility and stabilized the α -helical conformation through salt bridges along the helix surface and the guest residue X included natural amino acids³ and unnatural ones containing alkyl groups with various branching patterns, to test the effect on helix stability of steric constraints on side-chain conformation^{3,4}. Circular-dichroism spectra at 277 K and pH 7 are reported. For natural amino acids, the conformational preference parameters are similar but not identical to those determined by Baldwin and coworkers, and correlate well with other previous results⁵. The unnatural amino acids suggest that the branching pattern of alkyl side-chains has a very important effect on α -helix stability, as a result of steric interactions between side chain and main chain. Baldwin and coworkers have made similar observations^{8,9}.

What then can the spectator conclude? There is consensus that the effects of sequence context and tertiary interactions strongly influence helix formation. Yet most authors insist (occasionally perhaps protesting too much) that there must be a unique set of numbers that expresses the ideal helix-forming tendency of amino acids in monomeric oligopeptides, in the absence of complicating effects such as explicit side-chain interactions and variations in conditions of solvent and temperature. Those who accept this second premise will note the general but imperfect qualitative agreement about relative helix-promoting tendencies of amino acids, and will await additional experiments that, if the premise is true, must ultimately reconcile the differences among reported values of Zimm–Bragg parameters. Others may wonder whether so few parameters can really do so much for so many variations and combinations of amino acids under different conditions of solvent and temperature, and will find it possible to accept that many if not all of the results can be valid, if only for the systems from which they were derived. □

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The past comes alive

Bryan Sykes

"PiG Brings Home The Bacon on DNA." Thus was the readership of one newspaper alerted to the revelation that Henry VIII's pork chop came from a pig. Hardly a triumph of historical reconstruction this, but nonetheless an important step in the process of authenticating the amplification of DNA from all sorts of dead things. Everybody is having a go — and apparently succeeding — at getting DNA from museum specimens, dried insects, mummies, old bones and fossil plants and, quite rightly, the press loves it. A meeting* last month provided a further outlet for reporting these efforts.

It all began in 1984 when Russell Higuchi and his colleagues managed to clone mitochondrial DNA (mtDNA) sequences from a 140-year-old museum skin of quagga, an extinct beast which looks like a cross between a zebra and a horse¹. The following year Svante Pääbo reported his cloning from 5,000-year-old Egyptian mummy skin and liver². This was hard cloning from exotic sources. Access to the field was widened by the polymerase chain reaction and by the discovery of amplifiable DNA in old bone³, which forms the great bulk of surviving organic material from the past.

Generally, ancient DNA is in pretty bad shape. It comes in short pieces, looks crosslinked under the electron microscope and is damaged to the extent that bases are often oxidized or missing. The *Taq* polymerase copes with such poor quality material by skipping between overlapping extension products if no continuous segment is present, but it has the habit of substituting adenines when it jumps from one strand to the other (S. Pääbo, University of Munich). The amplification products thus contain random errors, which can be averaged out by direct sequencing, and are chimaeric in the sense that they are not copies of a single contiguous target. This does not matter if the target is haploid (mtDNA for instance), but for diploid and higher ploidy nuclear genomes, which are not uncommon in plants and fishes, the products might well be from more than one allele. This has led to complexities in reconstructing *HLA-DQ* haplotypes from the remarkably preserved human brains from the Windover peat pit in Florida (ref. 4; W. Hauswirth, University of Florida). The site contains 91 well-preserved brains from a total of 177 skeletons buried 6,000–8,000 years ago. By both dot-blotting of amplified *HLA-DQ* alleles with specific oligonuc-

leotides, and amplification of cytosine-adenine dinucleotide repeats in the *Apo-A2* gene, the same alleles have been detected in individuals buried 1,000 years, or about 50 generations, apart.

Unusually good preservation conditions, when a basalt flow dammed a steep wooded valley creating a deep anoxic lake which silted up rapidly, have produced the record for the oldest DNA so far. This was from a magnolia leaf between 17 and 20 million years old (ref. 5; E. Golenberg, Wayne State University). Leaves of other species from the same deposits, including cypress, oak and tulip tree, have also given DNA. The sequences of the chloroplast rubisco genes are recognizably specific and the results have now been verified in three different laboratories. This means these compression fossils defy the prediction, from *in vitro* estimates of the rate of spontaneous hydrolysis, that no DNA would remain intact much beyond 10,000 years. What a good job not everybody knew that, grant reviewers included.

The *Clarkia* fossil sequences from the Miocene are being used to construct their molecular phylogenies, and this is clearly an area in which ancient DNA will help tremendously. A nice example is the examination of the moas from New Zealand. There were eleven species of these flightless birds, ranging from 3 to 11 feet in height, and they were all

eliminated by AD 1500 after the arrival of the Polynesians. Sequences of 12S ribosomal RNA from bone and soft-tissue remnants from four of the eleven species showed them to be very closely related despite the considerable morphological differences, a phenomenon common in island populations, and not at all close to New Zealand's other living ratites, the three species of kiwi (A. Cooper, Victoria University, Wellington). Neither were the moas closely related to the Australasian emu and cassowary, which are in turn closer to the ostrich.

Dried skins from a very large museum collection of kangaroo rats (a topic covered in News and Views⁶) were used to bring home the point that the extent of variation needs to be matched by sample size if any sense is to be made of ancient population surveys (K. Thomas, University of California, Berkeley). Mitochondrial D-loop sequencing from rats collected between 1911 and 1930 defined 23 mtDNA haplotypes or 'mitotypes'. Present-day sampling from the same site showed some mitotypes had been replaced by others but, because of the large number of variants, this was still not sufficient to be able to say that gene frequencies had changed significantly. The problem will be a considerable one for all those attempting to interpret the genetic structure of past populations and draw conclusions about, for example, the maintenance of genetic diversity following population bottle necks. Projects to do just that with, at one extreme, the Hawaiian goose (R. Fleisher, Smithsonian Institute,

Jerry Young

IN December 1989 the skeletal remains of a young female murder victim were discovered by workmen in the garden of a terraced house in Cardiff, Wales. She had been dead for several years. With little more to go on than her hair and approximate age from dental records, a remarkable reconstruction led to the unequivocal identification of the girl by bone DNA typing, as described by Hageberg, Gray and Jeffreys on

page 427 of this issue. The initial breakthrough came from the facial reconstruction by Richard Neave, a medical artist at Manchester University. Neave made a plaster cast of the skull and then meticulously built up the facial muscle using modelling clay (left). In little more than a day, the model was complete (right). Two days after pictures of it were released a Cardiff social worker telephoned the police believing the victim to be Karen Price, who had run away from a children's home in July 1981. Although dental records strongly supported the identification, final confirmation came when Jeffreys and coworkers amplified and typed DNA from bone samples using the polymerase chain reaction (PCR). The results were entirely consistent with the victim being the daughter of the presumptive parents, and marked the first instance of PCR-derived forensic evidence being accepted by a British court. **Kevin Davies**

*Ancient DNA, University of Nottingham, 8–10 July 1991.

Washington, DC) and, at the other, Amerindians (A. Stone, Illinois State University, and Andrew Merryweather, University of Pittsburgh) are having to take that into account.

Genetic diversity in ancient maize has also been under investigation. The 3–5 per cent sequence divergence of the alcohol dehydrogenase (*Adh2*) gene among modern strains was once thought to be due to accelerated evolution since domestication began in Guatemala and Mexico about 10,000 years ago. Conventional molecular clock estimates for the mtDNA sequence change with time (about 1 per cent each million years) would place the divergence from a common ancestor, which was probably the very unmaize-like teosinte, at least two million years ago. But maize samples from a 4,000-year-old site in Peru show just as much sequence diversity as now, which implies that rather than modern maize being a direct descendant of any single ancestor, it is a monstrous hybrid (P. Goloubinoff, University of California, Berkeley).

Frequency estimates of inherited disease alleles feature on many people's list of objectives and the successful amplification of the cystic fibrosis locus, though not the common phenylalanine codon deletion at position 508, was reported from two laboratories (E. Birban and D. Turbon, University of Barcelona; S. Hummel, University of Göttingen). These are the first reports of single-copy nuclear gene recovery from bone. In the second case, the study was the positive control for a bone-sexing study. There is no sure way of determining the gender of juvenile skeletons from morphometric parameters and it is usually deduced from the grave goods. Resolving this archaeologically important issue has been an obvious target from the beginning but, even though amplifying Y chromosome sequences such as the *DYZ1* repeats in modern DNA is (in the words of one delegate) "like falling off a log", no laboratory has yet managed to sex a blind series of ancient adult bones that have been sexed morphometrically and come up with the right answer. Because such a study is about the only opportunity for independent validation of the DNA results, could the apparent failure to do so be due to contamination problems on a wider scale than hitherto appreciated? If so, how many other results, where independent corroboration is not possible, are also unreliable? The field must face this ghastly spectre

even if it means installing rigorous containment facilities, thereby making life difficult for low-budget operations, if it is to avoid a severe population bottleneck of its own.

The mood lifted after this sombre speculation with a description of specimens of the mosquito *Culex* from Tanzania

that are so well preserved that you can see the red blood in well-fed examples. Could these insects have bitten an early hominid? The reporters sharpened their pencils.

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ORE DEPOSITS

Ocean cycles in control

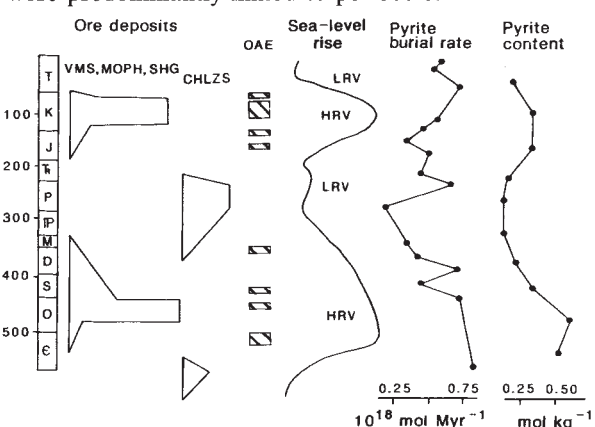
Ulrich Petersen

THE oxygen content of the oceans may seem to have little bearing on gold and metal-bearing ores. Yet in *Geology* (19, 645–648; 1991), Spencer R. Titley contends that the relation can be quite intimate. He argues that he can identify two classes of ores deposited during the past 600 million years (the Phanerozoic): those, including sedimentary-rock-hosted gold, formed 500–400 and 150–70 million years ago, at a time (he suggests) of, among other things, low-oxygen content; and those (clastic-hosted lead–zinc–silver deposits) layed down 270–225 million years ago (the Permian) at a time of high oxygen content.

The geographical distribution of ore deposits (commonly envisaged as in metallogenic provinces) and their apparent restriction to certain periods (the metallogenic epochs) have been debated for over a century. In the past, metallogenic provinces and epochs of ore deposits formed within the Earth's crust were predominantly linked to periods of

magmatic activity related to subduction and mountain building (as with porphyry copper–molybdenum deposits and many hydrothermal base- and precious-metal vein and replacement deposits), to continental rifting (carbonatites for example) or to the migration of mantle hot-spots (various magmatic ore deposits and kimberlite pipes). Alternatively, they were linked to times when crustal deformation led to the migration of brine from major basins (Mississippi Valley type lead–zinc deposits, for example) or to regions subjected to intense metamorphism (as with pegmatites).

Among the ores formed on the Earth's surface, the emphasis has been either on regions of favourable climate (for the formation of laterites, bauxite and supergene enrichment) or with suitable conditions for the mechanical accumulation of minerals in 'placers'. Times of special atmospheric composition have also been recognized, as for conglomeratic gold–uranium deposits. For sedimentary deposits (such as coal, petroleum source rocks, phosphorites and sedimentary iron/manganese deposits), the pertinent factor appeared to be the sedimentary environment or facies, although some periods seemed especially favourable to the development of several of these deposits (for example, the periods of deposition of banded iron formations, 3.8–1.8 and 0.9–0.7 billion years ago).



Titley's evidence for the part played by ocean oxygen in determining mineral deposits. The two classes he identifies comprise volcanogenic massive sulphides (VMS), metallized ophiolitic (MOPH) ores and sedimentary-rock-hosted gold (SHG) on the one hand, and clastic-hosted lead–zinc–silver deposits (CHLZS) on the other. The eons and their ages (in million years) are indicated on the left. Titley shows the periods of deposition for the first class to be correlated with oceanic anoxic events (OAE), which occur when the oxygen minimum zone is most extensive. These have been associated with the interruption of ocean bottom circulation by high ocean-ridge volumes (HRV, as opposed to low ridge volumes, LRV) which cause the sea level to rise. The burial rate and sedimentary-rock content of pyrite are also correlated with these changes.

There are, however, a number of metallic ore deposits for which both magmatic–hydrothermal and sedimentary factors may have been important. W. Klau and D. E. Large (*Geol. Jahrbuch* D40, 13–58; 1980) linked these deposits to (1) greenstone belts (2) felsic (silica-rich) volcanism; (3) mafic (magnesium–iron rich) volcanism; and (4) sediments. Titley now sug-

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gests that the presence or absence of these deposits may depend on the varying seawater compositions during the Phanerozoic (see figure). There are two ways the relation Titley identifies between deposition and anoxia can be viewed.

The first is to assume that certain seawater compositions favour the enrichment of marine sediments in certain elements. For example, gold and copper would accumulate preferentially during times of oceanic anoxic events associated with high sea levels, high oceanic-ridge volumes and high burial rates of pyrite. At other times, lead, zinc and silver would be concentrated in marine sediments. These sediments would then constitute the source rocks from which hydrothermal fluids would extract the metals to form the pertinent ore deposits.

The second is by assuming that the specialized oceanic compositions result in sediment compositions that are likely to precipitate certain metals preferentially from hydrothermal fluids passing

through them. Thus, sediments formed in a reducing environment would contain reduced minerals that would tend to precipitate gold and copper; those formed in more oxidizing environments would precipitate lead, zinc and silver.

These two hypotheses can be tested by analysing the pertinent sediments to see if their average contents of gold, copper, lead, zinc and silver differ as expected. Alternatively, laboratory experiments and thermodynamic calculations can test the idea of preferential deposition.

If true, the relations noted by Titley can be useful in mineral exploration because the search for specific metals can thus be directed to host rocks of certain ages, saving the expense of investigating host rocks less likely to contain the desired metals. It is, therefore, of practical importance to verify the relations and test the explanations. □

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ASTRONOMY

When big is beautiful

S. R. Kulkarni, M. Shao and C. A. Haniff

A YEAR before it has fully completed its first 10-metre telescope, billed as the largest in the world, the W. M. Keck Foundation has decided to fund a second, identical telescope only 80 metres away. But this is no sign of mere profligacy. Rather the idea is to take advantage of modern optics to use the two together as an infrared and optical interferometer to give astronomical images of unprecedented quality.

It has been remarked that the greatest progress in astronomy has depended more on new instruments than on the intellectual prowess of astronomers. Indeed, revolutionary discoveries have resulted from opening of new electromagnetic bands: radio, infrared and X-rays. Equally fundamental advances have resulted from high-angular-resolution imaging. This has been most evident at radio wavelengths, for which unprecedented milliarcsecond resolution has enabled astronomers to image blobs of gas as they are ejected at relativistic speeds from the enigmatic central sources of quasars, to determine geometrically the distance to selected objects in the Galaxy, and to measure the deflection of radio rays bent by the Sun's gravity. The Keck Foundation's decision is the latest in the many efforts to bring high-resolution imaging techniques to infrared and optical wavelengths.

The angular resolution of a telescope at a given wavelength is limited by the ratio of the wavelength to its diameter.

Milliarcsecond imaging requires telescopes with diameters ranging from nearly the diameter of Earth at radio wavelengths to hundreds of metres at optical and infrared wavelengths. Interferometers with a small number of modest telescopes separated by large distances (usually referred to as 'baselines') offer a

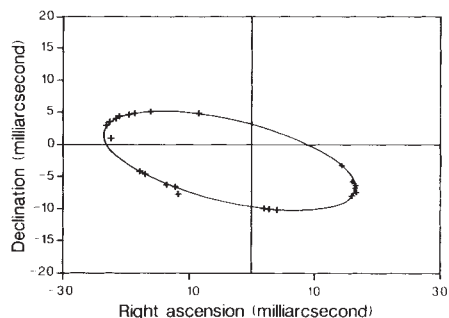


FIG. 1 The apparent orbit of the nearby binary stars Alpha Andromeda.

practical and cost-effective means of achieving high angular resolution. Examples include the Very Large Array, a radio interferometer consisting of 27 telescopes or 'elements' located in New Mexico, and the Very Long Baseline Interferometry (VLBI) network consisting of a heterogeneous collection of radio telescopes spread over several continents.

Even though Michelson and Pease pioneered astronomical amplitude interferometry at optical wavelengths almost 70 years ago, there was little progress till recently. Turbulence in the atmosphere

restricts the useful size of each element in the ensemble to several inches. Atmospheric refraction changes on timescales of several milliseconds and optics have to be controlled to a fraction of an optical wavelength. In contrast, at radio wavelengths, atmospheric effects are small and the wavelengths are five orders of magnitude larger, which is why radioastronomers were the first to benefit from interferometry. Fortunately, several new technologies (computer-aided servo control, laser metrology, precision optical components and mechanical design) have made optical interferometry feasible.

The small collecting area and short integration times of optical interferometric arrays limit their sensitivity to objects no fainter than one-tenth of the faintest naked-eye objects. The first modern interferometer was demonstrated by Labeyrie and coworkers in France. A group at Sydney University in Australia, an early pioneer, is nearing completion of a very long-baseline interferometer. In the United States, the Mark III Stellar Interferometer, a two-element device built by a consortium of four institutions, has been in routine operation since 1986, making well over 100 observations per night. The interferometer is already doing better astrometry than obtained by classical techniques and also has made precision measurements of several dozen binary systems (Fig. 1). Owing to the sensitivity limit, these interferometers are mostly pointed at bright stars: precision measurements of diameters, masses and distances and, of course, astrometry.

Fortunately, atmospheric turbulence is less of a problem at longer wavelengths. In the traditional infrared band of 2–10 μm the collecting areas and integration times are almost an order of magnitude larger. With the achievable sensitivities, a large, diverse group of objects — evolved stars, young stars with planetary disks, galaxies undergoing bursts of star formation, and so on — can be studied. Two-element infrared interferometers are now in use in France and the United States, mostly as demonstration units.

At optical and infrared wavelengths, and in radio VLBI, the rays reaching different elements are independently corrupted by the atmosphere which makes ordinary interferometric imaging impossible. However, the sum of the phases on a triplet of baselines which close to form a triangle, the so-called closure phase, can be shown to be immune to local atmospheric corruption (Fig. 2). Closure-phase imaging is one of several 'adaptive optics' imaging techniques which use the observed data themselves to measure and eliminate the atmosphere induced corruption. The technique is limited to bright objects but is otherwise very powerful. Indeed, such techniques are routine with VLBI where they are a

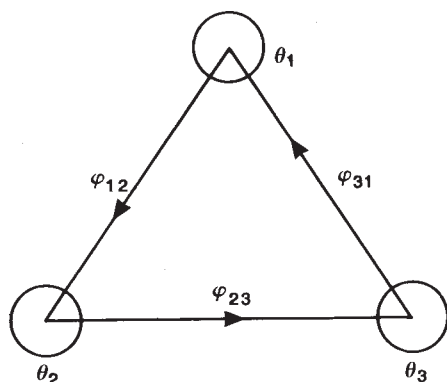


FIG. 2 Closure phase with a three-element interferometer. The interferometric phases are denoted by ϕ_{12} , ϕ_{23} and ϕ_{31} . Each element suffers from local atmospheric corruption (giving phases marked θ_1 , θ_2 , and θ_3). Because the difference of the atmospheric phases enters the interferometric phase, the closure phase — which is the sum of the three interferometric phases, taken in the order shown by the arrows — is independent of the atmospheric phases.

must and even with the VLA for making the highest-quality images by eliminating local errors.

Closure-phase imaging requires a minimum of three elements and benefits rapidly with increasing number of elements. The technique has been successfully demonstrated at optical wavelengths by using say, five to seven small portions (just a few inches across) of a large optical telescope to mimic the different interferometric elements. Application of radio VLBI software to such data has resulted in images at the limit of the resolution of the telescope (about 40 milliarcseconds) and with quality comparable to routine radio VLBI images. Several separated-element optical/infrared interferometers with three to six elements and resolutions approaching a few milliarcseconds are now in various stages of planning and construction.

The recent advent of adaptive optics as well as declassification of adaptive-optics technology by the US Department of Defense has made interferometry in general, and infrared interferometry in particular, more attractive than ever. Adaptive optics is similar to closure-phase imaging in that the signal from the source is used to correct for atmospheric errors, but it is done at the time of observing. Adaptive optics allows the use of apertures larger than set by the atmosphere. In particular, at a good site like Mauna Kea, the use of adaptive optics enables the use of the entire 10-m area of the Keck Telescope for 10- μm interferometry.

The construction of a second 10-m telescope only 80-m away from the first Keck telescope offers possibility of a very sensitive infrared interferometer with resolutions of 25 milliarcseconds at 10 μm and 5 milliarcseconds at 2 μm . The interferometer offers the possibility of directly

detecting warm Jupiter-sized planets in orbit around nearby stars. (Sadly, the wobble of the pulsar PSR1829-10, caused by the orbit of its newly discovered planet (M. Bailes *et al.* *Nature* 352, 311–313; 1991), is too small for any interferometry.) Young stars and their protoplanetary disks will be favourite targets as well. It should also be possible to place constraints on the size of the infrared-emitting regions of active galactic nuclei.

Although the addition of the second 10-m telescope will allow progress on these fronts, true imaging would require the addition of at least one more telescope and preferably more. As in radio VLBI, it is not necessary for all the telescopes to have the same size. Indeed, at the longer wavelengths, the sensitivity of a fully phased array is determined by the total collecting area.

The European Southern Observatory has ambitious plans for interferometry as well. The Very Large Telescope (VLT) project, which is fully funded, consists of four 8-m telescopes to be located in Paranal, Chile. Interferometry between the four telescopes as well as two to four additional 1.8-m telescopes is an explicit part of the VLT project. Thus by the end of this decade we may well have two rather powerful infrared interferometers in operation.

And the future? The recently released Bahcall report has recommended the construction of a large, VLA-style, optical/infrared array. Adaptive optics in conjunction with creation of 'artificial stars' by laser illumination of the upper atmosphere may allow the use of large elements and effectively overcome the sensitivity limit of closure phase techniques. The ultimate interferometer would be put in space. The absence of an atmosphere allows large apertures, long integration time, access to the ultraviolet region, freedom from thermal background (important at long infrared wavelengths) and, most importantly, precision astrometry. The Bahcall report recommends an interferometer with sensitivity to 20th magnitude objects and with astrometric accuracy between 10–30 microarcseconds. Such an instrument would give three orders of magnitude of improvement in accuracy and three to four orders of magnitude increase in sensitivity over the Hipparcos astrometric satellite. The scientific gains of such an interferometer will be truly enormous: distance scale, mass distribution, planet detection. While the astrometric gains are obvious, the gains in imaging, judging from the historical experience of high-resolution imaging, can be expected to be revolutionary as well. $\square$

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Deep cold

To a swimmer, the surface of the sea may not seem very warm; but the depths are colder still. At a depth of 800 m it is only 6 °C and dropping steadily. The deeps reach 2 °C. In theory, energy could be extracted from this thermal gradient. A floating thermal power station, with conventional turbines but a low-boiling working fluid, has been proposed. Daedalus now plans to use the oceanic temperature difference to power a novel submarine. It is simply an underwater glider, a sealed plastic envelope inflated into a wing-like hydrofoil. Cunningly, it is inflated with heavy water.

The 'heavy-waterglider' is ballasted to be slightly denser than surface seawater. It is towed out to sea by a tug, and simply released. It tilts nose-down and glides at a shallow angle into the depths. Now heavy water has an unusual property: below 11 °C it expands on cooling. At 3.8 °C it freezes, with even greater expansion. So as the heavy-waterglider sinks into the ocean and begins to cool down through this range, it slowly expands. Even if it doesn't freeze, it ultimately becomes less dense than the water around it. Its nose comes up, and it starts a buoyant glide upwards again. The steady climb towards the hotter surface soon warms it up: it begins to contract. At the surface it is warming and contracting so fast that it soon loses the last of its buoyancy. It then heads down into the frigid depths on a second 'vertical tack'. It will travel endlessly.

This wonderful thermal oscillator will need careful design. It must glide efficiently enough to travel a useful distance on each tack; its thermal inertia and rate of heat flow must be chosen to switch it rapidly between strong positive and strong negative buoyancy at the top and bottom limits of its trajectory. The heavy water may need modifying, by mixing in other solvents.

Automatic navigation is another problem. An electronic steering package, powered by a small propeller projecting into the craft's slipstream, will maintain a compass course, updated by a satellite 'fix' at each rise to the surface. As the craft approaches its destination, it will be guided to rendezvous with the receiving tug by sonar steering commands.

Oceanographers will love the heavy-waterglider. Fleets of these cheap, simple vehicles will carry instruments and take samples over huge tracts of deep ocean and long periods of time, and return them safely to port. Their brief enigmatic appearances on the surface, and occasional encounters with fishing nets, will fuel new nautical tall stories about sea-monsters. Trials are being conducted in Loch Ness.

DAVID JONES

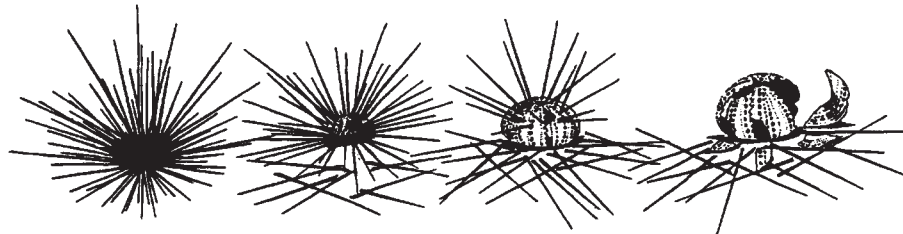
Threat to black sea urchins

SIR — Many sea urchins are dying in the Florida Keys, in Puerto Rico and possibly elsewhere. Information about the locations and extent of this problem is urgently needed to understand this major disturbance.

In 1981, masses of diademid sea urchins throughout the Hawaiian islands died. The phenomenon was never

Atlantic and among similar diademid in the Pacific. A mass mortality of the commercially important West Indian sea egg (*Triplaneustes esculentus*), for example, has just begun in Puerto Rico. If any readers can help, please send details to E.H.W.

The limited information available suggests that these new outbreaks are



Stages in the death of a sea urchin.

studied¹. From early 1983 to the beginning of 1984, 95–99 per cent of all of the ecologically important black longspined sea urchins (*Diadema antillarum*) perished throughout the western North Atlantic, the largest and most devastating mortality of an invertebrate ever recorded². New outbreaks of this disturbance occurred in Panama, St Croix and possibly other locations in the Caribbean in the autumn of 1985³.

In 1990, while coral reef bleaching was occurring in these areas⁴, we learned of mass mortalities of the urchins in Jamaica, the Cayman Islands and Belize. Each mortality of urchins occurred over a period of approximately one week, killing most of the remaining *D. antillarum*. We organized a working group to determine the cause of the deaths. Circumstantial evidence implicates an infectious pathogen², possibly a virus⁵ or a bacterium.

Although we were unable to obtain moribund urchin specimens from the 1990 events, masses of urchins died at the beginning of May near Key West, Florida, mortalities spreading slowly up the Keys. We have obtained adequate specimens for microbial studies, but we need additional field observations of the disease signs, date of occurrence, locality, speed of progress of the disease, approximate numbers of animals affected, date on which last mortalities were seen, weather conditions, temperature, and so on.

Although our attention is focused on the Florida Keys, this disturbance may also be occurring in other areas of the

progressing more slowly (6–8 days in a location, rather than 3–4 days in 1983–84), but they remain just as lethal to *D. antillarum*. It is to be hoped that cooperative efforts will demonstrate a cause for the disease and enable us to understand this ecological disturbance.

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Lead risks

SIR — Several US regulatory agencies are considering the health risks to children of low levels of lead in blood (less than 20 µg per 100 ml). There is considerable debate about how to set acceptable levels. Bellinger, for example, has summarized¹ several studies reporting data on IQ and lead in blood and teeth from several countries. The graphs in his paper have been widely circulated as key summaries of recent research. We are concerned, however, that the figures do not accurately represent the primary sources and thus may provide inappropriate policy guidelines.

Because lead exposure and IQ scores are both correlated with socioeconomic variables, these parameters have to be taken into account when estimating the effect of lead on IQ. But although Bellinger's Fig. 2 legend states that the data plotted are mean adjusted IQ scores, it is the raw, unadjusted data from ref. 2 which are in fact plotted. This is impor-

tant because confounding bivariate were found to account for half of the apparent effect. For Fig. 3, Bellinger bypassed the overall results in favour of plotting the raw data for one subgroup only. Similar distinctions apply to the data from refs 3–6 which are also plotted in the Bellinger figures.

We agree with Bellinger that the results from individual studies need to be put into a wider context. But it should be made clear, especially to policymakers, that the two summary graphs in ref. 1 cannot be used as the basis for regulatory decisions. Moreover, the actual lack of consistency across the studies suggests that the policy options being considered require further examination.

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Refractive lens for X-ray focus

SIR — The European Synchrotron Radiation Facility, the Advanced Photon Source in the United States and the Super Photon Ring in Japan will provide high-brilliance X-rays with very long beam lines of up to 1 km. To use the long beam line, we have designed a small-angle X-ray scattering camera, the small-angle resolution of which is as good as 10 µrad. We recognize the difficulty that the beam should be reduced to 1 mm in diameter and be focused at 1 km away from the light source. Conventional focusing devices such as totally reflecting mirrors require a stability of the order of 1 µrad for the angle of reflection.

Here, we propose the use of refractive lenses which we believe have been overlooked for focusing X-rays¹. The refractive index n of materials for X-rays is less than unity: $n = 1 - \delta$; δ is of the order of 10^{-6} for an X-ray of 0.1 nm in wavelength, depending on the elements and density of the material². Therefore, we can use a concave lens to focus X-rays at long distances. The table

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Material, radius of curvature (r) and focal length (f) of biconcave lens for 8-keV X-ray

Material	r (mm)	f (m)
Gold	10	100
Gold	5	50
Platinum	10	90
Tungsten	10	100

shows the materials, the radii of curvature and the focal lengths for biconcave lenses. Because the lens is concave, we can reduce the thickness to a few micrometres by using a Fresnel lens, which can be made with an ultra-fine lathe. The intensity reduction due to absorption, therefore, may be a factor of only 1/10. Parasitic, fluorescent X-rays from the lens diverge isotropically; we can eliminate them by conventional slits.

The advantages of lenses over mirrors are as follows. (1) The instability of the principal axis of the lens relative to the beam direction does not give rise to serious change in the position of the focal image of the light source. (2) The parasitic scattering due to the surface roughness of a lens is reduced to a factor of δ (10^{-6}) compared with that for a mirror with the same roughness. (3) The lens is much smaller than a mirror; to get a beam 1 mm in diameter, a totally reflecting mirror is typically 10 cm in length, whereas a lens needs to be only a few millimetres in diameter. No bending

mechanism is required for lenses. (4) As the lens is made of metal, cooling efficiency is high. This is an important feature of optical devices for strong synchrotron radiation. (5) The beam path is straight in the lens system; a totally reflecting mirror causes deviation of the focused position from the straight beam path by a few tens of metres for a 1-km beam line.

Another possible application of X-ray lenses may be microscopy. For soft X-rays of 10 nm in wavelength, δ is about 10^{-2} for graphite; we can adopt concave lenses for X-ray microscopy instead of zone plates.

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Bayesian reasoning in science

SIR — C. Howson and P. Urbach in their Commentary on bayesian reasoning in science (*Nature* 350, 371-374; 1991) state: "It might be imagined that a 95 per cent confidence interval corresponds to a 0.95 probability that the unknown parameter lies in the confidence range. But in the classical approach μ is not a random variable, and so has no probability. Nevertheless, statisticians regularly say that one can be '95 per cent confident' that the parameter lies in the confidence interval."

It seems that this problem can be solved by saying: the 95 per cent confidence interval covers μ with a probability of 95 per cent.

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SIR — Howson and Urbach's eloquent contribution¹ to the literature intended to persuade scientists to adopt bayesian methods of statistical inference does not fall on deaf ears, but rather on ears eager to hear any fresh argument that could dispel the doubts repeatedly expressed in the 140 years since the time of

George Boole. For it is certainly true that, were the universal application of bayesian methods possible, both statistical theory and statistical practice would be greatly simplified. But Howson and Urbach do nothing to dispel these doubts, which can be summarized as follows.

No reason has been advanced for why the calculus of probability, so perfectly matched to the treatment of uncertain events, should apply to the treatment of scientific hypotheses. Whereas in the former case, the universe of possibilities is closed under the logical operations of 'and', 'or' and 'not', in the latter case this is not true, so that the addition axiom of probability is not appropriate. It was the removal of this axiom by R. A. Fisher in 1921 which gave rise to the important non-additive concept of likelihood.

Nor is it correct to imply, as do Howson and Urbach, that the application of the modern axioms of probability to hypotheses derives from the work of Fermat, Pascal, Huyghens and Bernoulli. Their work grew out of games of chance; even when Bernoulli, in the fourth part of *Ars conjectandi*, turned his attention to the problem of statistical

inference, the only example he gave of the application of his famous (non-bayesian) procedure involved drawing balls out of an urn. Nor should the name of Thomas Bayes be too closely associated with modern bayesian arguments: that cautious man not only (like Bernoulli) made no attempt to publish his work during his lifetime, but his advocacy of the use of a prior distribution was tentative.

Unsurprisingly, the application of the probability axioms to scientific hypotheses leads to serious difficulties concerning the prior distributions to be adopted in the absence of information, as may be demonstrated using an example of Fisher's. A homozygous black mouse (*BB*) when mated to a heterozygous black (*Bb*) will produce only black mice, but half will be expected to be homozygotes and half heterozygotes. An offspring of such a mating, therefore, may be stated, before progeny-testing, to be homozygous with probability one-half.

Now a bayesian statistician, confronted with a black mouse about whose ancestry he knows nothing (and whose ignorance of mendelian genetics is, in any case, complete) is constrained by his axioms to assert that, because he is unable to see any reason why the black mouse should be of one genotype rather than the other, the mouse is homozygous with probability one-half. Thus the bayesian's assertion, based on ignorance, is exactly the same as the scientist's, based on partial knowledge.

Fisher² remarked that "It is evidently easier for the practitioner of natural science to recognize the difference between knowing and not knowing than this seems to be for the more abstract mathematician", adding that "no experimenter would feel he had a warrant for arguing as if he knew that of which in fact he was ignorant". As Boole³ had remarked in 1854, the appearance of arbitrary constants reflecting the prior assumptions of bayesian theory "seems to imply, that definite solution is impossible, and to mark the point where inquiry ought to stop".

Though I hold no brief for the 'repeated-sampling' theories of statistical inference about which Howson and Urbach are critical, in one example they are unfairly so. They assert that statisticians "never say why one can be '95 per cent confident' that the parameter lies in the confidence interval" in the case of the unknown mean μ of a normal distribution with known (not estimated) standard deviation of the mean s and observed mean m , for which the confidence interval is $m - 1.96s \leq \mu \leq m + 1.96s$.

The reason was given by Fisher in the early 1930s. He pointed out (1) that if

such a statement is made whenever this type of problem arises it will be true precisely 95 per cent of the time; (2) the mean of a normal sample, being technically a 'sufficient' statistic, incorporates all the available relevant information in the sample; and (3) complete absence of knowledge *a priori* about μ ensures the validity of the 95 per cent probability statement on this particular occasion of use. The statement has exactly the same logical content as the statement "This penny, which I have just tossed but which is still hidden on the back of my hand, has probability one-half of being heads."

Howson and Urbach repeat Neyman's old *non-sequitur*, that in the classical approach a parameter is not a random variable and therefore cannot be the

subject of a probability statement. The case of the already-tossed penny proves this false. Bayesians have no exclusive claim to probabilities for parameters — fiducial theory has been there already, as the example given above indicates; but their claim that all parametric uncertainty can be measured by probabilities will remain in doubt until compelling arguments are advanced and scientists' misgivings thus removed. This is probably not possible.

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Tumbling controls bean weevils

SIR — The larvae of the common bean weevil, *Acanthoscelides obtectus* (Say), bore into and develop inside bean (*Phaseolus vulgaris* L.) seeds. In tropical conditions, the adults emerge within one month of seed infestation, whereupon each female lays about 60 eggs loosely among the beans¹. Lack of effective controls for exploding *A. obtectus* populations severely limits bean storage by tropical subsistence farmers². Human hardship is caused both by direct loss of the chief dietary source of lysine and leucine, requisite supplements to a mainly cereal diet, and by indirect losses to poor seed germination.

Video analysis of *A. obtectus* host-colonization behaviour³ reveals that the mobile 0.2-mm diameter by 0.6-mm-long first instar larvae bore into beans only where they can wedge themselves between a bean and some stable, resistive surface such as another bean or the walls of a container. Apparently, this leverage enables the larvae to direct sufficient forces for their mouthparts to scrape

through the tough seedcoat of a dried bean. Remarkably, it takes 24–48 hours of nearly continuous scraping for a larva to enter the cotyledon of a red kidney bean³. Larval mortality is appreciable during entry, but not thereafter.

We wondered if moving the start of an *A. obtectus* entrance hole out of register with bracing sites would render that hole useless. Based on measurements of beans and attacking larvae, we calculated that there would be only a 0.04 probability that, after bean tumbling, the start of a hole would fall back into register for continued use. We postulated that larvae displaced by bean tumbling would be unlikely to find the rare, realigned entrance hole and be forced to start boring anew. With regular tumbling of beans, the larvae would die of exhaustion or be smashed.

This hypothesis was tested using a paired design with half-filled 0.8-litre glass jars, 16-litre plastic food buckets and burlap bags containing, respectively, 0.24, 7.0 and 22.7 kg of intact, dried red kidney beans (*P. vulgaris*, L.) and 30, 480 and 1,000 *A. obtectus* adults. After introduction of the beetles, jars and

buckets were rolled two circumferences every 8 hours for two weeks; similarly, bags were twice tumbled end over end two to three times a day. Control containers were fixed in place throughout the experiment.

Populations of *A. obtectus* in all rolled or tumbled containers were reduced dramatically (by around 97 per cent) relative to those in stationary controls (see table). This level of control was remarkably close to the 96 per cent predicted from calculations of the effect of moving holes out of register before their completion. But as some smashed eggs and larvae were seen on the walls of the glass jars, multiple sources of mortality probably contributed to the overall population reduction.

We recommend tumbling dried beans morning and evening as a practical control of *A. obtectus* populations available immediately to anyone storing beans in clay pots, baskets, jars, tins or bags. This 'technology' is highly appropriate in developing nations as it requires only the transfer of knowledge, unlike controls that rely on chemical application or specialized equipment. Moreover, this control principle might be applicable to other bean and grain pests whose requirements for seed penetration are temporally and spatially constrained. The possible benefits of regularly moving beans and grain in commercial bulk storages should also be considered. Although some attention has been paid to this idea for grain^{4,5}, attempts at mechanical control of pests in storage need to be backed by more information on pest biology, including behaviour. (A fuller version of this study is to appear in *Entomol. exp. appl.*; details are available from M.E.Q.)

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REDUCTION OF *A. OBTECTUS* POPULATIONS AS A RESULT OF CONTAINER ROLLING.

Container/ Treatment	<i>n</i>	No. beetles introduced	Mean no. beetles emerging
0.8-l Glass jar			
Rolled	10	30	21 ± 15*
Stationary	10	30	575 ± 130
16-l Plastic bucket			
Rolled	2	480	171 (160; 182)†
Stationary	2	480	7,622 (6,812; 8,431)
45-kg Burlap sack			
Tumbled	2	1,000	244 (228; 259)
Stationary	2	1,000	6,857 (5,101; 8,612)‡

* Mean ± s.d.

† Beetle counts for both trials are given in parentheses.

‡ Reduction across the experiment was significant at $P < 0.001$ according to paired *t*-test on log transformed data.

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Odd omission

SIR — Benton (*Nature* **352**, 113; 1991) provides useful information on the natural dispersion and habitat of rubbish. But how can he account for the remarkable absence of supermarket shopping trolleys and condoms in his sample?

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Terrestrial primordial neon

SIR — In their letter¹ about neon and helium isotopes at Loihi and Kilauea (Hawaii), Honda *et al.* published data similar to ours. But their interpretation is not as strongly supported by their data as they claim. In their Fig. 1, Honda *et al.*¹ plot many more data than listed, and most of the data are indeed atmospheric within error. The *L-K* correlation line is established using all of the data. One consequence is to force the regression line through the atmospheric point. But in the table, they removed those data that are atmospheric within two standard deviations, hence discarding both the data with large errors, but also those with small errors which plot close to the atmospheric composition.

Their Fig. 1 shows only six anomalous points, meaning that they plot above the mid-ocean-ridge basalt (MORB) area established by us², most of which have large errors. Moreover, consideration of only these points shows that they can be fitted by a line parallel to the mass fractionation line (mfl). Mass fractionation is thus likely, and further confirmed by considering the duplicate analyses for samples KK29-4 and KK1712: the data are clearly different for both aliquots, and precisely plot on lines parallel to the mass discrimination line. This is shown in Fig. 1 here, where we plot total-gas data for the eight samples in the table of Honda *et al.* It is important to realize that mass fractionation can operate on non-atmospheric gas (see, for example, ref. 3).

The rest of the dispersion can be accounted for by mixing between

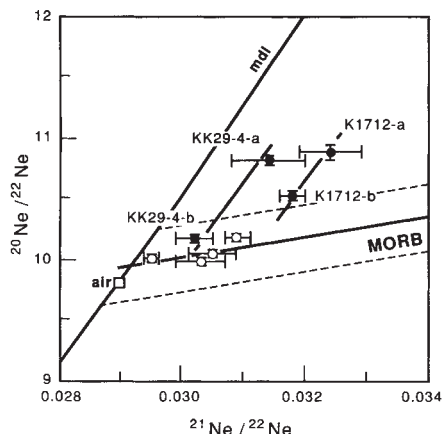


FIG. 1 Total gas neon data for all samples listed in ref. 1, plotted in the three-isotope neon diagram. Filled circles represent two duplicate analyses for samples KK29-4 and Kilauea 1712. The duplicate data lie on lines parallel to the mass discrimination line (mdl). Mixing is especially manifest for samples represented by the empty circles, while mass discrimination is likely for samples represented by the filled circles.

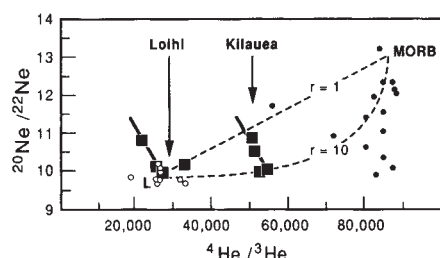


FIG. 2 Helium-neon isotope diagram. Filled circles, data for MORB; open circles, data for Loihi from ref. 2; squares, data for Loihi and Kilauea from ref. 1. Mixing plus mass fractionation is manifest, especially for samples from Kilauea. Broken lines, mixing curves.

atmospheric-like neon (from the undegassed mantle) and degassed mantle-like neon (MORB), as for five points in our Fig. 1, which plot within the MORB area. Mixing has already been observed at Hawaii by Kurz *et al.*⁴, who used helium isotopes. For Loihi, mixing may be due to MORB neon from xenocrysts, present in alkali Loihi samples⁵, and shown to release MORB helium upon heating. We showed² that the anomalous neon appears together with enhanced ⁴⁰Ar/³⁶Ar isotope ratios, which is a strong argument in favour of a MORB component, but not of a solar origin. Confirmation of the above interpretation comes from the ²⁰Ne/²²Ne-⁴He/³He diagram (Fig. 2), where Kilauea data¹ fill the gap between Loihi and MORB. Both data sets show alignments with the same negative slopes, which can be quantitatively attributed to a single stage mass fractionation.

Altogether, some of the data in ref. 1 can be explained by mixing combined with mass fractionation, but most of them are indeed isotopically atmospheric. The authors' conclusion that primordial terrestrial neon was isotopically solar is entirely based on their poorly defined *L-K* line, and is therefore not strongly supported by the data, which, conversely, show clear mass fractionation effects which Honda *et al.* unfortunately did not discuss.

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HONDA *ET AL.* REPLY — Sarda and Staudacher argue that variation in observed neon isotope ratios in the Hawaiian samples could be attributed to recent isotope fractionation along paths parallel to the mass fractionation line (mfl), labelled mdl in Fig. 1. However, they disregard the fact that isotope frac-

tionation must be accompanied by concomitant element fractionation. As we clearly stated¹, the calculated ratio of nucleogenic ²¹Ne to radiogenic ⁴He in most of the Hawaiian samples listed in our table is close to the theoretical production ratio. Because the slope of the mixing line between solar and atmospheric components is not very different from that of the mfl, the amount of nucleogenic ²¹Ne is effectively calculated as the deviation from the mfl line passing through the atmospheric composition. Thus, calculation of the nucleogenic ²¹Ne/radiogenic ⁴He ratio is essentially the same irrespective of which model is chosen to account for the high ²⁰Ne/²²Ne ratios in the Hawaiian samples. The similarity between the theoretical production ratio and the calculated nucleogenic ²¹Ne/radiogenic ⁴He ratio in the samples is regarded as strong evidence that there has been no substantial element fractionation between helium and neon. As isotope fractionation is likely to occur only under physical conditions more extreme than those leading to element fractionation, this apparent lack of element fractionation suggests that isotope fractionation of neon is unlikely to have occurred.

We have now shown⁶ that there are additional and systematic correlations between the isotope compositions of neon and helium in terrestrial samples, including basaltic glasses from both plume (Hawaii) and mid-ocean-ridge situations, gas samples from the continental hydrothermal system associated with the Yellowstone hotspot and ancient Zaire diamonds. The solar hypothesis successfully accounts for helium and neon isotope systematics of all available samples, irrespective of sample type, tectonic setting and age, providing us with additional confidence that the Earth's primordial noble-gas composition contains a solar component.

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Extraordinary archaeology

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The Davenport Conspiracy Revisited. By Marshall McKusick. Iowa State University Press: 1991. Pp.206. \$22.95.

In 1877, diggers wrenched from an archaeological mound near Davenport, Iowa, two slate tablets incised with concentric circles, signs of the zodiac and other remarkable designs. The excavators soon found other objects, including a concretion with quartz eyes, skeletal material and a limestone tablet on which a pudgy, red-painted, human figure with an enigmatic smile was represented straddling the Sun and holding a bow: it was said to be a 'mound builder'. At a later date, effigy pipes came into the diggers' possession. Soon, the archaeological and antiquarian world was ablaze with speculation about these artefacts. Even *Nature* saw fit to comment, in 1885, that "the whole subject is of extraordinary interest", and admitted that if the remains were authentic, they would "have much influence on the final settlement of the question of who the Mound Builders were".

McKusick's book is about these remains, the question of their authenticity, and the conspiracy to conceal the fact that they were faked. The lines were clearly drawn: on one side were sceptics based mainly in the east at institutions like the Smithsonian, who argued that the tablets were not genuine; and on the other side were most of the members of the Davenport Academy, and others, who proclaimed that the tablets were made by the same people whose remains were found in the mounds — the mound builders — who were not ancestors of contemporary native Americans.

McKusick treats the Davenport conspiracy like a whodunnit. He draws widely on published, archival and oral evidence to discuss competently, if occasionally in turgid prose, the details of the conspiracy, to trace its contours, and to plumb the motives of the many characters involved. The cast of characters included many who might have been involved in forgery: a wealthy Yankee lawyer and benefactor of the local scientific academy, several amateur archaeologists, a man known as a stonemason, a janitor clever at carving pipes, and a recent German-speaking immigrant from Switzerland who was the pastor of a Lutheran church and an indefatigable digger (and, in the looseness of the day's archaeology, 'looter') of mounds. All fell under suspicion at one time or another. All had some

motive for forgery. Some were committed to the theory that mound builders came from somewhere in the Old World. One needed money. One wanted respectability for Davenport and mistrusted the scientific establishment in the east. One was the butt of ethnic jokes. As with any whodunnit, it would be unfair to give



Hard to swallow — the limestone tablet showing the Iowa mound builder. The eyes of both effigy pipes depicted at the top were glued on with white cement; the eyes were subsequently lost.

away the ending here, except to say that the systemic explanation provided by McKusick is reasonable, even if his language occasionally betrays his uncertainty about the origins of the conspiracy.

Why has McKusick revisited this conspiracy? In 1970, he published *The Davenport Conspiracy* (University of Iowa Press) in which, one might assume, he settled the issue to his own satisfaction. But not everyone, it seems, agreed with his interpretation. It may seem remarkable that anyone today would doubt that the mound builders were, in fact, the ancestors of contemporary native Americans and that the tablet mak-

ers were nineteenth-century European-Americans, but at least one scientist argued in the 1970s that one of the tablets was the American equivalent of the Rosetta stone! Unfortunately published widely, his preposterous theories struck a responsive chord among a public too ready to believe that American Indians did not possess the ability to create certain architectural features, or that many features of the landscape were produced by extraterrestrial visitors, or both.

And so McKusick has re-issued this book as a response to these recent developments. He has made certain changes from the earlier edition so that it might appeal to a larger audience, and he has added a chapter to refute the speculations of Barry Fell, a charismatic cult archaeologist whose ideas crumble owing to their lack of logic yet remain appealing to the public.

But despite these changes and additions, I cannot help feeling that McKusick has not taken full advantage of the opportunity to engage more fully with some of the most interesting aspects of the Davenport conspiracy. These have to do, first, with the ways that European-Americans constructed each other in the late nineteenth century — there was a dispute in Davenport between German-speaking immigrants and established Yankees — and second, with the ways that European-Americans collectively constructed others like native Americans, who in the dominant model of the day, were firmly positioned at a lower rung on the evolutionary ladder. Another interesting point concerns the widespread tendency to produce fakes, either for monetary gain, to play a trick, for intellectual deception, or for some other end.

Archaeological interpretation that allows for mound builder theories was not unique to nineteenth-century America; in Africa, for example, missionaries, explorers and some ethnologists attributed sub-Saharan Africa's complex technology and society to people of Mediterranean or Egyptian origin. In America, from the late eighteenth through to the late nineteenth century, scepticism that native Americans were of sufficient sophistication to produce certain archaeological features was rife, and belief in an extinct race of mound builders strong. In different hands, the mound builders were Scandinavian Vikings, Welshmen, Hindus, Egyptians, Phoenicians, Hittites, or a lost tribe of Israel — virtually anyone but American Indians.

As forgeries go, the case of the Davenport tablets was not nearly as significant as that of Piltdown Man — to mention perhaps the best known

palaeontological/archaeological fraud. But it is most revealing of the cultural sentiments of entire generations of European-Americans: Yankee versus recent immigrant, whites against what they perceived as the culturally unsophisticated American Indians. It was indeed a case of "extraordinary interest". □

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Science matters

Kenneth Green

The Management of Science. Edited by Douglas Hague. Macmillan/St Martin's Press: 1991. Pp. 180. Hbk £40/\$59.95; pbk £14.99.

THIS collection was first presented at the 1989 meeting of the British Association for the Advancement of Science. The nine papers fall into two groups: five are based on economic, historical and sociological research in the growing field of 'science policy studies'; four are by 'practitioners', recently or currently involved in shaping, setting and administering scientific budgets. Unfortunately, there are no contributions from any industrial practitioner of research management — so the 'management' being referred to concerns state-funded research, almost all of it to be carried out in universities or government laboratories. Management is also being considered mainly at the strategic level — of research-council organization and national decision making — rather than at the operation level.

Nevertheless, taken together, the papers give a quick look into the arguments behind the debate on the future of scientific research in Britain. The practitioners' contributions are, perhaps inevitably, idiosyncratic and anecdotal. Douglas Hague is especially provocative; as one-time chairman of the UK Economic and Social Research Council, he has some rude things to say about the scientist-administrators he came across in his dealings with other research councils in the mid-1980s. They did not necessarily share his enthusiasm for the applications of Hague's personal specialism — managerial economics — to planning and steering their scientific budgets; nor did they take kindly to Hague's espousal of 'corporate planning' of research-council business. Frederick Dainton, chairman at one time or another of many British science policy-making bodies, offers a sharp retort to Hague's managerialism. In a ponderous article, he refers to management techniques "insidiously filtering down, even

Face from the past — still clad in the sealskin parka it was wrapped in for its journey to the land of the dead, this six-month old baby has been preserved in excellent condition for around 500 years, thanks to the low ground-temperature and dry air at the site of its grave, at Qilakitsoq on the west coast of Greenland. In about 1475, six women, a young child and this baby were buried in the traditional way — warmly clothed and provided with goods for their journey. Their graves were excavated in 1978, and the mummies were dated to within 50 years either side of 1475, making them the oldest known find of well-preserved humans in Greenland. *The Greenland Mummies*, edited by J. P. H. Hansen, J. Meldgaard and J. Nordquist, tells the story of the investigation into how they died, a question that is still unresolved, and discusses the customs surrounding death and burial in their society. Published by British Museum Publications, price is £14.95.



to basic research".

The Hague-Dainton 'debate' is, I fear, about the wrong things. Certainly, science has to be 'managed' and 'corporate planning' will have its place. But what precisely should be the substance of the plans? The 'research' papers suggest a few things, many of them not mentioned by or even in opposition to the views of the practitioners. For example, Keith Pavitt, of Sussex University's Science Policy Research Unit, disputes the notion that managing science is now necessary just because funding resources have become scarce, with science supposedly in a 'steady state'. It is true that publicly funded basic research in Britain has been growing very slowly in the 1980s, but not in other countries.

All but one of the papers are about the management of science in Britain. This is both a strength and a weakness: a strength because the book gives British scientists and policy-makers lots to think about; a weakness because little is said about how our more successful economic rivals actually manage their science.

Whether non-British readers will find much to illuminate their own problems, I doubt, because, as far as the management of science goes, Britain is a peculiar country. It is indeed true that the growth rate of Britain's government-funded spending on scientific research is the lowest of the rich countries. Yet, as Margaret Sharp shows, in some research fields, such as those related to biotechnology, its quality-research productivity (measured by citations) is second only to that of the United States and well ahead of those of Germany and France. The

UK research system is also the least 'self-contained', the most open to 'exploitation' by non-British firms. More than 20 per cent of UK patents taken out in the United States are taken out by foreign firms' UK subsidiaries (only Belgium and Canada are more dominated by foreign transnationals). Further, as Paul Stoneman reports, the most recent figures show that at least 12 per cent of UK industrial research and development is paid for by funds that come from overseas. Will the twenty-first century see Britain as an off-shore floating research-and-development laboratory for foreign transnationals?

Even our political governance of science is strange: John Krige points out how Britain's long-established (and continuing) aversion to 'Europe' means that it contributes to the international nuclear physics organization, CERN, exclusively from domestic research funds. Both the French and the Italians, however, see CERN as a political gesture and thus fund it through their foreign ministries.

All these peculiarities should, I suggest, be the subject of national political debate about the organization and funding of research and the setting of national priorities. The book is full of policy suggestions to keep student seminars and laboratory common rooms buzzing. But new forms of funding and organization are not enough. As Brian Wynne points out, in his analysis of how Cumbrian sheepfarmers reacted to the (everchanging) prognostications of agricultural scientists on the longevity of Chernobyl fall-out, lack of public interest in the funding problems of science in Britain

might well be a reflection of failures of the scientists themselves. It is not that the public does not understand science, he argues, rather that it has learnt not always to trust the opinions of scientists: "science is in a negative, if slowly unfolding, spiral of the self-destruction of its own public credibility." Now, how is corporate planning going to deal with that? □

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■ Also for those concerned about the prospects of science in Britain comes *British Science and Politics Since 1945* by Tom Wilkie. Published by Blackwell, this addition to the series 'Making Contemporary Britain' charts the history of British science and scientists in relation to government policy. Price is £29.95 (hbk), £8.95 (pbk). □

Living together

Brian Charlesworth

Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis. Edited by Lynn Margulis and René Fester. MIT Press: 1991. Pp. 408. \$37.50, £33.75.

IT IS now universally accepted by biologists that the mitochondria and chloroplasts of eukaryotic cells are the descendants of bacteria-like organisms that took up residence within host cells, establishing an 'endosymbiotic' relationship. There is suggestive evidence from recent work on *Chlamydomonas* that the same is true of the centriolar apparatus. Lynn Margulis has played a large part in persuading biologists that such important features of eukaryote cells are the evolutionary product of endosymbiosis, and her contributions to this subject have provided a valuable addition to our understanding of the evolution of life. The purpose of this symposium volume is to bring together contributions from a diverse array of biologists to discuss whether symbiosis plays a major role in the generation of evolutionary novelty.

Symbiosis is defined here by Margulis as "association through a significant portion of the life history" between members of two different species. As many of the papers document, there is a wide range of examples of symbiosis, involving very different levels of intimacy between the partners. At one extreme, there are the mitochondria and chloroplasts, the genomes of which are much reduced in complexity compared with their ancestors and which are dependent

on the host cell for many of the functions necessary for their survival. At an intermediate level, there are essentially obligate associations between partners, either intracellular or extracellular, such as the endomycorrhizal fungi associated with the roots of many species of flowering plants. In other cases, one of the partners can be found either as free-living or in association with the host, and the host has to be reinfected each generation. An example of this is the photobacterium associated with the light organs of gadiform fish. It is often difficult to distinguish between a parasitic and a mutualistic relationship, and critical data on fitness costs and benefits are often lacking. The start of a mutualism may often involve an exploitative relationship; conversely, mutualistic relationships may evolve into parasitic ones.

Two very different perspectives on the significance of symbiosis for general evolutionary biology are represented in this book. One, expounded lucidly by Richard Law and John Maynard Smith, is that symbiosis represents an interesting and important element in evolution, but in no sense offers a challenge to neodarwinian evolutionary theory. The other, vehemently stated by Margulis in the first chapter and espoused to varying extents by Robert Haynes, David Bermudes and Richard Back, Paul Nardon and Anne-Marie Grenier, Werner Schwemmler and Kris Pirozynski, is that neodarwinism is seriously incomplete, and that "the current practices of population biology and genetics must be obliterated by their own false assumptions" (Margulis, page 11). According to Margulis: "The standard textbooks on evolution catechize [sic] all species and higher taxa (genera, families, phyla) as having evolved in the same way: by the gradual accumulation of favourable mutations. Yet not a single example of the origin of such lower taxa (species) exists in the literature. Rather, the highest taxa (kingdoms and phyla) have evolved by acquisition of symbionts that have become hereditary."

This claim, if true, would certainly require a considerable reappraisal of evolutionary theory. There are, however, substantial grounds for doubting that it contains more than a grain of truth. In the first place, it is clear that symbiosis can allow a species to acquire a set of functionally integrated, adaptively useful characters in one step; for example, species of fish and nematodes have become luminescent by acquiring bacteria with elaborate pathways for light production. But unless a system of Chinese boxes is allowed, the ultimate source of the characters acquired by the host must be through the conventional darwinian process of the stepwise accumulation of individually advantageous changes.

Second, it is abundantly clear from many examples of symbiosis that the initial association sets the stage for a series of evolutionary adjustments by both partners. Again, there is no reason to suppose that this is anything other than a purely darwinian process. (One disappointing aspect of the book is the lack of discussion of the causes of some of these secondary events; for example, why should the nuclear genome have taken over so many of the functions of mitochondria and chloroplasts?) The properties conferred on the host by the original symbiosis were probably very different from those observed today.

Third, it is far from clear that symbiotic events in themselves are causally involved in either the splitting of lineages, or the evolution of the suite of characters that defines a major group. At the lowest level, the acquisition of a component of the gut flora by a species of insect or vertebrate does not in itself constitute the origin of a new species, unless it is accompanied by reproductive isolation from the rest of the population. Although there are some examples of hybrid sterility induced by endosymbionts, the evidence strongly supports the importance of nuclear genes in most cases of reproductive isolation that are susceptible to genetic analysis. At higher levels, there is no evidence that evolutionary events that are usually considered to be major are due to symbiosis: it seems unlikely, for instance, that the notochord was acquired in this way. Even though modern eukaryote cells contain endosymbionts of critical importance to their functioning, it is a mistake to identify the origin of the taxa concerned simply with the acquisition of the symbionts in question. Eukaryote cells, after all, have numerous important characteristics in addition to mitochondria. Darwinian evolution is the only tenable explanation of how such combinations of multiple characters can be built up.

The strength of this book is in the wealth of information on the natural history of symbiosis. Evolutionary biologists will certainly profit from this, although there are certain blind spots, such as the omission of any discussion of entities such as retroviruses and transposable elements, which surely meet Margulis's criterion for symbiosis. Its weakness lies in the exaggerated claims for symbiosis as the major source of evolutionary novelty. This may provoke unnecessary reluctance among mainstream evolutionists to think about the questions raised by symbiosis, which would surely be a pity. □

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In the bag

J. J. D. Greenwood

Bird Trapping and Bird Banding. By Hans Bub. Cornell University Press: 1991. Pp. 330. \$69.50.

CATCHING birds has long been a matter of art and ingenuity. Many of the traps invented have been capable of capturing birds alive, which has allowed birds to be taken into captivity. The great diversity of trapping methods that has developed has been taken over by bird-ringers ('banders' in American English), who aim to catch and mark the birds for the purposes of scientific study and in whose hands trapping methods have developed and multiplied yet further.

This book presents an exhaustive review of the ways of catching birds alive. It will be useful both to the experienced ringer looking for new methods to aid his pursuit and to the beginner looking perhaps to find away of trapping the species that he has taken as the subject of his doctoral research. Unfortunately, it has several major deficiencies. The first is that its title is misleading: it contains virtually no information on banding or on the activities that commonly accompany banding, such as distinguishing age and sex, taking measurements, recording moult and breeding condition, scoring fat deposition, and taking blood samples and parasites.

The problem facing the author of a manual of trapping methods is that one could choose to organize it by method, by species to be caught or by habitat in which one is operating. None would be ideal: a careful blend is probably required, backed with a good index. Bub's presentation is, unfortunately, a jumble, in which the reader gets no aid from the index, which contains entries for species of birds only. On the finer scale, the precise ways of making and operating particular traps are too often obscure. Although the book is abundantly illustrated, many of the illustrations are not illuminating. In some of the line drawings it is impossible to see how the trap is constructed, let alone how it works. And many of the photographs do not show the necessary detail, which is rendered even more of a problem by their frequent murkiness.

Although the book presents a huge number of methods, it makes too few attempts to assess their relative merits. The beginner, especially, is likely to be overwhelmed, unable to sort the gold from the dross. This is compounded by the idiosyncrasy of the practical hints that are given, with trivial or self-evident points being made while important questions of practice are often not men-

tioned. In discussing mist-nets, for example, it is suggested that one should colour-code the loops on the shelf-strings, but there is no consideration given to the requirement to guy the nets properly or to the question of how much lengthwise tension there should be on the shelf-strings or how much vertical slack there should be in them.

Proper guying of nets is partly a matter of safety for the birds, which are endangered if a net collapses. It is on the welfare of the birds that the book is weakest. The forewords by George Jonkel and Chris Mead point out that the techniques described may be risky and that many of them may be illegal in some countries, but the book pays scant attention to this. Indeed, it encourages undesirable practices, such as laying holding-bags with birds in them on the ground, and putting more than one bird in a bag. Bags, we are told, should be washed 'from time to time': my experience is that they should be washed

every time they are used, not only because clean and dry bags are better for the birds, but also because dirty bags, shedding the dust of dried faeces as they are opened, are bad for the ringer. (The section on dangers to the bird-bander covers matters such as tree climbing, but none of the problems of picking up *Campylobacter* or other infections.)

Bird welfare is important not only in its own right and in terms of public relations, but also because any technique that harms the birds jeopardizes the quality of the science that is the ultimate objective of the ringing; so failure to address it is a serious deficiency in the book. But not withstanding its deficiencies, the diversity of methods presented makes it a useful addition to the ornithological library — provided that it carries the label 'use with care'. □

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Nuclear free

Gerald E. Brown

Nuclear and Particle Physics. By M.S.C. Williams. Oxford University Press: 1991. Pp. 385. Hbk £40, \$83; pbk £19.95, \$32.50.

CHARM pervades this text, designed "for use by university undergraduates in the penultimate year of their studies for a first degree in physics". Williams begins by sketching the developments around the turn of the century that gave rise to atomic and nuclear physics, complete with lovely photographs of the protagonists and enlivened by cartoons. There are few problems for the student, possibly because Williams has adopted the Oxford style of teaching, in which there is considerable interchange between tutor and student.

The uncouth Americans, on the other hand, in their nearest equivalent (*Subatomic Physics*, second edition by Hans Frauenfelder and Ernst M. Henley: Prentice Hall, 1991) launch straight into particle accelerators. Frauenfelder and Henley believe that the important tools are necessary from the start, and they absolutely plaster the student with problems.

Both books cover some astrophysics including supernova 1987A, the Big Bang and nucleosynthesis. There are a few errors in Williams' book: the Chandrasekhar mass is $5.76Y^2M_\odot$ not $1.2-2M_\odot$ as in the text. Nevertheless, Williams' qualitative discussions of astrophysics are accurate.

My criticism of the book is that the treatment of nuclear physics is woefully

out of date by about two decades. No wonder nuclear physics research in Britain is being turned off! Superdeformation is not mentioned; the beautiful treatments of the monopole, dipole, quadrupole and Gamow-Teller giant resonances are skipped; and the giant dipole resonance is barely alluded to. The important interplay between the nuclear many-body problem and those in condensed matter physics is not developed. The study of matter under extreme conditions, high temperature and high density, is not even mentioned, although — with the development of SIS at Darmstadt, the AGS heavy-ion accelerator at Brookhaven, the planning there for the relativistic heavy-ion collider, and the 200-GeV/nucleon beams at CERN — this is now the main thrust of nuclear physics.

According to Williams, "the problem is that there is no unified theory of nuclear reactions that is of practical use." He goes on: "The theories of nuclear structure and behaviour do not form a unified model in the sense that all properties can be predicted or explained from a set of assumptions of a fundamental nature. Nuclei are complicated many-body objects held together by poorly understood forces. . . ." I am left speechless. I am used to physicists in other fields being ignorant of nuclear physics, and also to their making comments like those of Williams — but I am not used to their writing textbooks on nuclear physics. Frauenfelder and Henley do much better! □

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Double giant resonances in atomic nuclei

S. Mordechai and C. Fred Moore

Giant resonances are collective excitations of the nucleus in which a large fraction of the component nucleons are set into coherent modes of vibration. Double giant resonances, in which vibration is excited in a nucleus already undergoing a giant resonance, were predicted thirty years ago, but have only recently been observed experimentally. Their properties confirm broad aspects of nuclear structure theory, but show some anomalies that have yet to be explained.

THE best-known example of a giant resonance is the giant dipole resonance (GDR), excited when γ -rays are absorbed by the nucleus. The electric field of an incident γ -ray exerts a force on the positively charged protons in the nucleus, causing them to move in relation to the uncharged neutrons. The frequency of the vibration between neutrons and protons, which can be imagined as two fluids, is governed by the strong nuclear interaction, which provides the restoring force¹.

Giant resonances can also be excited by weak or strong interactions, and are a general and well studied property of all nuclei. Since 1971, giant resonances of other multiplicities, such as E0 (monopole), E2 (quadrupole) and E3 (octupole), have been obtained experimentally, mostly in inelastic hadron and electron scattering experiments. The resonances are further classified into isoscalar (labelled $T=0$), in which the whole nuclear medium of neutrons and protons oscillates in concert, and isovector ($T=1$), in which neutron and proton oscillations are in antiphase. Figure 1 illustrates the geometry of the lowest three multipoles of isovector modes. A third type of resonance involves magnetic vibrations, in which the two antiphase components are nucleons with spin up and those with spin down. These resonances have recently been observed, but are discussed later in this review only briefly. In the Brink-Axel hypothesis, advanced in the 1950s^{2,3}, it was assumed that the basic properties of giant resonances (their strength and frequency) are governed purely by the characteristics of the nuclear force, independent of whether the nucleus is in an excited state or not. This meant that it should be possible to find giant resonances built on highly excited or "hot" nuclei, with large angular momenta. Figure 2 is a schematic energy-level diagram illustrating the Brink-Axel hypothesis for giant dipole excitations. The left-hand side shows the ground state ($|gs\rangle$) and first few excited states ($|1\rangle, |2\rangle, \dots$) of a nucleus, and the right-hand side shows the energy levels of GDRs built on these states. If D is the giant dipole operator, then the Brink-Axel hypothesis is that $|D; i\rangle$ turns state $|i\rangle$ into a GDR with energy increased by the constant amount $\hbar\omega_{\text{dipole}}$, where ω_{dipole} is the giant dipole frequency.

Until a few years ago, GDR states could be created only from nuclear ground states, but the development of heavy-ion accelerators has allowed experimental verification of the Brink-Axel hypothesis⁴. But studies have also shown some remarkable general differences between GDRs built on excited states and those on ground states⁵.

Of special interest is the case when the excited state on which the GDR is built is also a giant resonance. The possibility of such double giant resonances was first pointed out by N. Auerbach⁶ as a new kind of collective nuclear excitation. They constitute a fundamental test of our understanding of collective nuclear phenomena and the basic structure of giant multipole resonances. If such double giant resonances exist, the fundamental question is whether their excitation energy, total width, integrated strength and other defining characteristics can be understood in terms of the properties of the single giant resonance. In nuclear structure theory, double giant resonances should be understandable from both the macroscopic and micro-

scopic points of view. In the macroscopic approach, the resonance is described in terms of collective motions of multinucleon states, whereas in the microscopic picture the wavefunction of the resonance is built up from two-particle, two-hole configurations (that is, two nucleons are excited to a higher unoccupied orbital in the nucleus, leaving behind two nucleon holes).

Double nuclear resonances have not been observed until very recently because of the experimental difficulty of observing a resonance of considerable width located at very high excitation energy, and superimposed on a large background from the continuum of states encountered in the nucleus at this background energy. In this review we describe the methods behind new experiments at the Los Alamos meson physics facility (LAMPF) to search for double giant resonances, the puzzles that have been solved and the new puzzles that have arisen.

Research at 'meson factories'

In the past 15 years, three 'meson factories' in Switzerland (PSI), Canada (TRIUMF) and the United States (LAMPF) have become fully operational and enabled extensive pion-scattering studies of the nucleus to be carried out. LAMPF produces one of the most intense pion beams, allowing the measurement of reactions with extremely small cross-sections. As a probe of the nucleus, the pion has several important advantages. One unique advantage is that it has three charge states: π^+ , π^0 and π^- , allowing a rich variety of experiments to be performed: inelastic scattering (with π^+ or π^-); single charge exchange (SCX) involving the reaction (π^+ , π^0) or (π^-, π^0); and double charge exchange (DCX), with (π^+ , π^-) or (π^-, π^+). In the notation (a, b), a is the incoming and b is the outgoing particle. The reactions are so called because of the charges exchanged between the nucleus and pions. Another dominant feature in pion scattering is the existence of a prominent pion-nucleon resonance, with a mass of 1,232 MeV, termed the $\Delta_{3,3}$ or the delta (3,3) resonance. At resonance (100–300 MeV of kinetic energy for the incoming pion) the elastic scattering cross-sections π^+-p (π^--n) and π^--p (π^+-n) are in the ratio nine to one. Therefore π^+ is more sensitive to protons and π^- to neutrons. It was found⁷ that a comparison of π^+ and π^- inelastic scattering provides

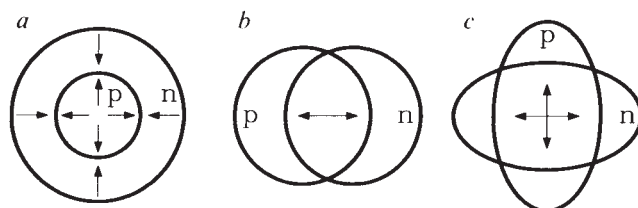


FIG. 1 The lowest three orders of isovector nuclear oscillations. *a*, In the giant isovector monopole, protons and neutrons 'breathe' in and out in antiphase. *b*, The isovector dipole, generally called the giant dipole resonance. *c*, The giant isovector quadrupole (IVQ). The corresponding isoscalar modes have the same geometry, but the neutrons and protons move in phase. In all cases the frequency of vibration is determined by nuclear compressibility, which is the restoring force.

an ideal probe for determining the relative contribution of neutrons and protons to an excited nuclear state, and the idea was successfully used for determining their relative contributions to inelastic transitions.

In SCX, one of the important observations after the construction of the π^0 spectrometer at LAMPF was the discovery of the isovector monopole resonance⁸ illustrated schematically in Fig. 1a. Two other, previously known, resonances have been surprisingly strongly observed in (π^+ , π^0) SCX. These are the giant-dipole resonance (GDR) and the isobaric analogue state (IAS). The IAS has essentially identical spatial and spin structure to the parent nucleus and is obtained simply by changing one of the excess neutrons in the target nucleus into a proton without any rearrangement of the other nucleons in the nucleus. The 'excess neutrons' are those outside the $N=Z$ core (N is the number of neutrons and Z the number of protons). They are also called 'valence neutrons' by analogy with chemistry's 'valence electrons'. The IAS was first observed in the (p , n) reaction and since then has played a unique part in nuclear physics. Both resonances (the IAS and the GDR) show up as the most prominent features in the SCX spectra^{9,10}. Pion SCX is ideally suited for exciting these resonances, because at the $\Delta_{3,2}$ resonance energies, the pion as a strongly interacting probe penetrates only slightly into the nuclear interior and probes mainly the outer region of the nucleus, where both the GDR and the IAS have strong components of their transition density.

Double charge exchange

In the simplest model, pion double-charge exchange reactions are viewed as two sequential SCX processes in which two neutrons are changed into protons, or vice versa. For target nuclei with $N-Z \geq 1$ the (π^+ , π^0) SCX process strongly excites the GDR and the IAS transitions. Therefore a combination of these resonances could, in principle, be reached in DCX. Figure 3 shows the isovector transitions and the energies involved in exciting these new double resonances, as well as the previously well-known double isobaric analogue state (DIAS). The DIAS is similar to the IAS: it is analogous to the original ground state except that two of the excess neutrons in the target nucleus are changed to protons in the same orbitals without rearranging the other nucleons. The ground states of the parent nucleus, the IAS and the DIAS are states which belong to the same isospin multiplet: that is, they have the same isospin quantum number

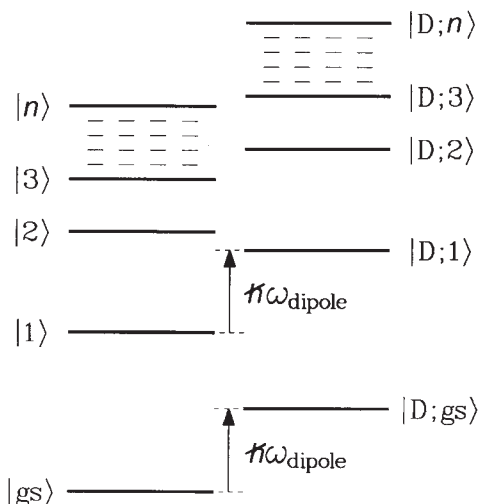


FIG. 2 Schematic nuclear energy-level diagram illustrating the Brink-Axel hypothesis^{2,3}. The figure shows the ordinary nuclear states (left) and the giant-dipole excitations built on the ordinary ground state (right).

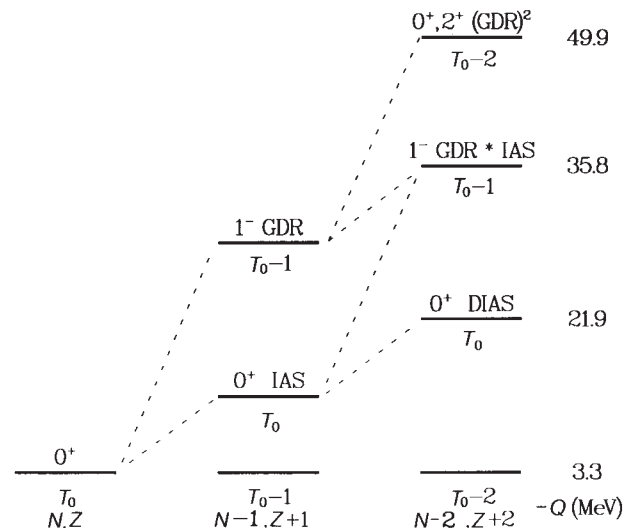


FIG. 3 Schematic energy-level diagram of 'single' and 'double' resonances anticipated in pion single-charge-exchange (π^+ , π^0) and pion double-charge-exchange (π^+ , π^-) reactions, respectively. The numbers to the right are the Q values for the ground state and the three double resonances observed in the DCX experiment on ^{93}Nb .

T , and differ only in T_z because of the change of one or two neutrons into protons, as discussed above. A simple example is ^{18}O (gs), ^{18}F (1.04 MeV) IAS and ^{18}Ne (gs) DIAS. (The isospin quantum number, originally called 'isotopic spin' or 'isobaric spin', was introduced to indicate that neutrons and protons have the same nuclear force and are similar in all respects except charge. Therefore they are treated as two possible quantum states, with $T_z = \pm 1/2$, of one heavy particle, the nucleon with $T = 1/2$.)

Figure 3 shows a schematic energy-level diagram of the prominent transitions observable in SCX and DCX. The lowest three double resonances expected in DCX are the DIAS, the giant dipole built on the isobaric analogue (or vice versa; we refer to both of these as $\text{GDR} \otimes \text{IAS}$) and the double isovector giant-dipole resonance (GDR^2). Only the lowest isospin component of the higher two resonances is labelled. The Q values listed in the figure refer to the case of ^{93}Nb discussed below. An example of pion DCX as an excellent tool to study double resonances was the observation of the well-known DIAS transition¹¹, which can be viewed in this context as the simplest double-resonance state. Until recently^{12,13}, none of the higher double resonances had been observed. For target nuclei with $J^\pi = 0^+$, the $\text{GDR} \otimes \text{IAS}$ is a single state with $J^\pi = 1^-$, where J is the total angular momentum and π is the parity of the state. On the other hand, the double dipole will have two possible spin values, 0^+ and 2^+ . The resonances also have different isospin components, but for heavy nuclei with a large neutron excess ($N-Z \gg 1$), the lowest isospin component is the dominant one.

Measurements

The measurements were performed at the energetic pion channel and spectrometer at Los Alamos using a special set-up for pion DCX¹⁴. This spectrometer has good background rejection and precise energy calibration and therefore was most suitable for these studies. The spectrometer can detect outgoing pions with a kinetic energy range of 100–300 MeV with an energy resolution of ~ 140 keV, and is considered one of the best pion spectrometers in the world. Electrons and muons (arising from pion decay in the channel or within the spectrometer path length) were identified and rejected using a freon-gas Cherenkov detector and a set of scintillators placed behind a series of graphite

blocks, respectively. Further details on the experimental set-up can be found in refs 15 and 16.

The GDR built on the IAS

Figure 4 shows typical Q -value spectra obtained from pion DCX in a recent experiment at LAMPF to search for double resonances. By analogy with radioactive decay, the Q value is defined as the difference between the initial and final nuclear masses. It is often expressed in energy units (such as MeV) in accord with special relativity. As π^+ and π^- have exactly the same mass, in the present reaction the negative of the Q value gives the amount of energy put into the nucleus to form a given state. The figure shows the results on three different targets, ^{13}C , ^{27}Al and ^{59}Co . The measurements were taken at an incident pion kinetic energy of 295 MeV (ref. 15). All three spectra show clearly the existence of a wide resonance located in the continuum region at ~ 8.8 and 14.2 MeV above the ground state of ^{13}O and ^{27}P final nuclei, respectively. On the ^{59}Co target the resonance is seen at ~ 16.8 MeV above the DIAS in ^{59}Cu (27.9 MeV above the ground state of ^{59}Cu). The resonances are identified as the giant-dipole resonances built on the isobaric analogue states or vice versa (GDR $\otimes$ IAS). The identification

is based on their energies, which are very close to the energy where the giant dipoles built on the isobaric analogue states are expected to appear, on the characteristic dipole angular distribution of the resonance, and on the cross-sections. Similar observations of the resonance have been confirmed on several other nuclei, indicating that this collective mode is a general feature of all nuclei that have at least one excess neutron¹⁵. The ground state, the DIAS and the resonances were fitted with a standard peak shape. The background which arises from DCX to the continuum, was fitted using a third-order polynomial. The energies and widths were varied simultaneously to minimize χ^2 for the entire fit. The figure shows clearly that the width of the GDR $\otimes$ IAS increases significantly with mass. For example, the resonance for ^{13}C has a width of 3.0 ± 0.6 MeV, but that for ^{59}Co has a width of 7.0 ± 1.0 MeV (more than twice as wide as for ^{13}C). The increase of the width with mass is not yet fully understood, and is one of the puzzles arising from these studies. Further details on these resonances can be found in ref. 15, where some systematic features of the GDR $\otimes$ IAS are outlined. The results indicate that in many respects (for example, energies, cross-sections and variations of the cross-sections with mass) the GDR $\otimes$ IAS does behave as a superposition of the two ingredient resonances, the GDR and the IAS.

The double giant dipole

We now turn to the observation of the double dipole. Figure 5 presents Q -value histograms measured for the $^{93}\text{Nb}(\pi^+, \pi^-)^{93}\text{Tc}$ reaction at 295 MeV at three different scattering angles. These spectra were taken in long DCX measurements in order to accumulate good statistics. They also cover a high excitation energy. The DIAS appears at $Q = -21.9$ MeV and is clearest in the 5° spectrum. In addition to the DIAS, two wider peaks are apparent in the spectra. The peaks are labelled GDR $\otimes$ IAS (GR1) and GDR $\otimes$ GDR (GR2). The first appears at

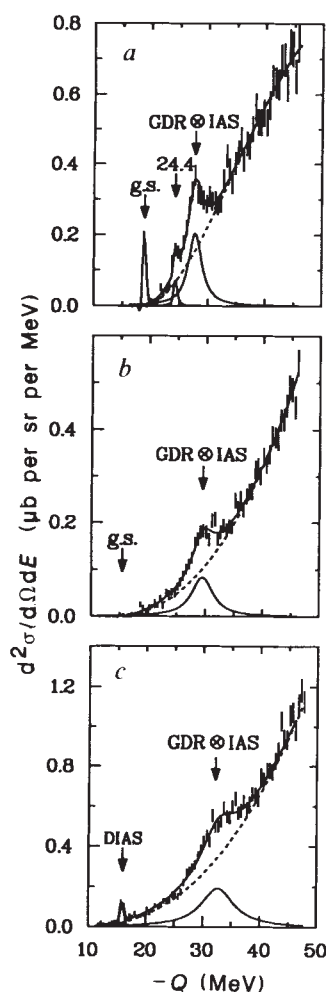


FIG. 4 Q -value spectra for (π^+, π^-) pion double-charge-exchange reactions at $T_\pi = 295$ MeV on three targets. *a*, $^{13}\text{C}(\pi^+, \pi^-)^{13}\text{O}$ at $\theta_{\text{lab}} = 18^\circ$; *b*, $^{27}\text{Al}(\pi^+, \pi^-)^{27}\text{P}$ at $\theta_{\text{lab}} = 15^\circ$; *c*, $^{59}\text{Co}(\pi^+, \pi^-)^{59}\text{Cu}$ at $\theta_{\text{lab}} = 11^\circ$. The arrows indicate the fitted location of the ground state, the DIAS and the giant resonance (GDR $\otimes$ IAS). Short vertical lines represent statistical uncertainties of the data. The dashed lines are the fitted background with a polynomial fitting code NEWFIT.

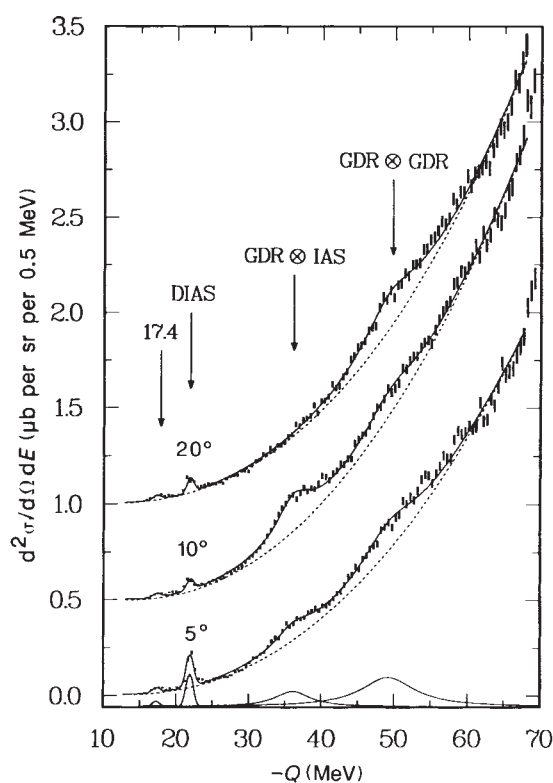
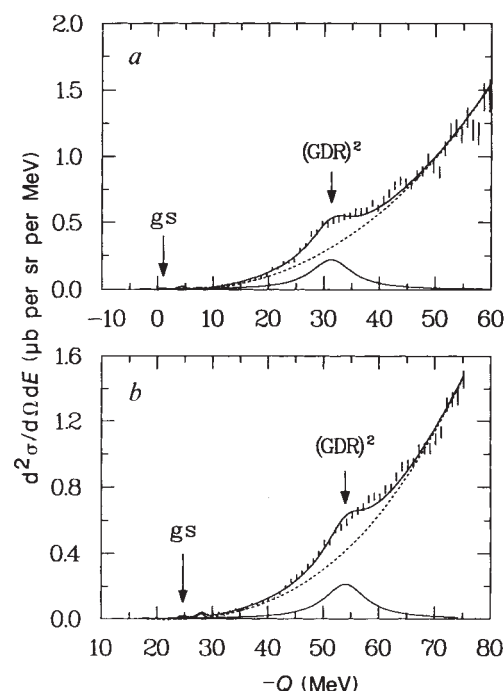


FIG. 5 Doubly differential cross-section for the (π^+, π^-) reaction on ^{93}Nb target ($^{93}\text{Nb}(\pi^+, \pi^-)^{93}\text{Tc}$) at $T_\pi = 295$ MeV and $\theta_{\text{lab}} = 5^\circ, 10^\circ$ and 20° . The arrows indicate the three double resonances observable in pion DCX: the DIAS, the GDR $\otimes$ IAS and the GDR $\otimes$ GDR.

FIG. 6 *a*, Doubly differential cross-section spectrum for the (π^-, π^+) reaction on ^{40}Ca ($^{40}\text{Ca}(\pi^-, \pi^+)^{40}\text{Ar}$) at $T_\pi = 295$ MeV and $\theta_{\text{lab}} = 5^\circ$. The arrows indicate the fitted location of the ground state (gs) and the giant resonance (GDR)². Short vertical lines represent statistical uncertainties of the data. The dashed line is the fitted background with a polynomial shape and the solid line is the fit to the spectrum. *b*, Same as (*a*) except for the inverse $^{40}\text{Ca}(\pi^+, \pi^-)^{40}\text{Ti}$ reaction.



$Q \approx -35.8$ MeV and the latter at -49.9 MeV. The fit shown uses $\Gamma_{\text{DIAS}} = 0.8$ MeV, $\Gamma_{\text{GR1}} = 5.8$ MeV, and $\Gamma_{\text{GR2}} = 8.8$ MeV. At 20° , GR1 vanishes, but it shows up clearly at 10° . The peak labelled GDR $\otimes$ GDR has a different angular distribution. Full angular distributions for the resonances have been measured on ^{40}Ca , ^{93}Nb and ^{56}Fe (ref. 16). Detailed analysis shows that the two resonances have different natures because they have a significantly different angular dependence. GR1 has a dipole shape and corresponds to the GDR $\otimes$ IAS discussed earlier. The higher resonance GR2 has a quadrupole distribution. From its energy (which is about twice the energy of the one-step GDR), angular distribution and cross section, we identify GR2 as the double giant dipole¹⁶. The double dipole has a width of ~ 8 – 10 MeV, which is larger than the width of the 'single' dipole by a factor of 1.5–2.0. This agrees well with theoretical estimates for the width of the double dipole based on two-phonon states^{16,17}.

A unique feature of the pion DCX reaction is the simplicity with which one can study the inverse reaction $((\pi^-, \pi^+)$ instead of (π^+, π^-)) on the same target nucleus. Experimentally, this is done simply by inverting the polarities of all bending and focusing magnets in the pion channel and the spectrometer. Recently (H. Ward *et al.*, manuscript in preparation), we used this feature at LAMPF to examine further the nature of the double giant dipole resonance. The (π^-, π^+) measurements are more difficult than the (π^+, π^-) , as π^- beam fluxes are generally smaller than the π^+ fluxes by a factor of five. Furthermore, the cross-sections involved are smaller (for $N > Z$ nuclei) in the (π^-, π^+) reactions than in the (π^+, π^-) reactions, making the running time for a single measurement significantly longer. Figure 6 shows the simplest case, in which the (π^+, π^-) and the (π^-, π^+) reactions were measured under the same experimental conditions on a self-conjugate ($N = Z$) nucleus. As the target nucleus has $N = Z$, the final nuclei reached in the two reactions are mirror nuclei (one is changed into the other by exchanging protons and neutrons). Because the nucleon–nucleon force is charge-symmetric, one would expect the double dipoles to appear at the same excitation energy with respect to the individual final ground states. They should, however, appear at significantly different energies (Q values) with respect to the original target-nucleus ground state, since in (π^+, π^-) we add two Coulomb energies and in (π^-, π^+) we subtract two Coulomb energies. Thus, the difference in the Q values of the

double dipole in the two reactions should be equal to four Coulomb energies minus four times the neutron–proton mass difference (1.29 MeV). For heavy nuclei this effect will considerably lower the double dipole. The resonances labelled (GDR)² in Fig. 6 were identified from their energies, angular dependence, widths and cross-sections as the double giant dipole resonances in ^{40}Ar and ^{40}Ti mirror nuclei. In the (π^+, π^-) reaction, the resonance is observed at $Q = -54.0$ MeV, but in the inverse reaction the same resonance appears at much lower energy, around $Q = -31.1$ MeV. The energy difference between the two reaction modes is in excellent agreement with the above predictions and provides a strong support for the identification of the double giant dipole state. To reduce uncertainties in the extracted parameters of the resonances, the same background form was used in both reactions. The fits shown in the spectra of Fig. 6 use a width of 9.0 MeV for the two reactions. The double dipoles in both DCX modes show the same characteristic angular distribution. The cross-section in the (π^-, π^+) reaction is $\sim 20\%$ larger than in the (π^+, π^-) reaction. This result is consistent with the SCX data for the 'single' GDR¹⁸ as well as with simple theoretical predictions. Consistent results have also been obtained on non-self-conjugate nuclei. In this case the analysis is more complicated, but valuable information on the isospin structure of the double giant dipole can be deduced from the data.

Summary and prospects

The two new double resonances reported here are the giant dipole built on the isobaric analogue state and the double giant dipole resonance. The identification of the resonances is based on the energies at which they appear, their angular distributions and their cross-sections. The data indicate that the giant dipole built on the isobaric analogue is a general collective feature of all nuclei with $N - Z \geq 1$, and the double dipole is a general feature of all nuclei. The observed double resonances behave as a superposition of the ingredient 'ordinary' resonances. But some puzzles arise from these studies. Why does the width of the GDR $\otimes$ IAS increase rapidly with mass, and where is the missing strength of the 0^+ member of the double giant dipole? Further experiments are planned at LAMPF to study these questions and the possible implications of these new phenomena. There is the possibility that other double giant

resonances, unobserved so far, could be detected. The isovector monopole resonance shown schematically in Fig. 1a was observed in pion SCX, and it is not inconceivable that the isovector monopole built on the IAS, or the double isovector monopole resonance, could be detected.

The new resonances reported here do not include any spin degrees of freedom. The giant Gamow-Teller (a transition which requires a spin-flip of a nucleon in the nucleus) was observed in the 1970s in the (p,n) reaction, and since then has been intensively studied in several laboratories. Much of the recent interest in the giant Gamow-Teller transition stems from the puzzle that only ~50% of the theoretical strength is observed experimentally. What are the prospects for detecting the double giant Gamow-Teller in nuclei? The existence of this new mode

was recently predicted theoretically^{19,20}, but attempts at experimental study have not been successful so far. The observation of the double giant Gamow-Teller will present a new challenging problem for nuclear structure studies, and its relation to double β -decay is of interest to both particle and nuclear physicists. Theoretically, many questions related to the nuclear structure, deformation effects, isospin splitting, widths and decay modes of the double resonance are still open. $\square$

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Fluxes and excess temperatures of mantle plumes inferred from their interaction with migrating mid-ocean ridges

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The behaviour of thermal plumes in the Earth's upper mantle is strongly affected by their interaction with nearby mid-ocean ridges. The magnitude of the buoyant topography and the length of the geochemical anomaly induced by plumes at migrating ridge axes provide a way to estimate their excess temperature and discharge rate, and thereby constrain their depth of origin.

It is now generally accepted that the Earth's mantle is convecting. The different scales and styles of convection, however, remain uncertain¹⁻³. Broadly speaking, the convective structure of the mantle is composed of at least two thermal boundary layers where horizontal flow dominates: one at the core-mantle boundary, the other just beneath the lithospheric plates near the Earth's surface. Some assume that a third thermal boundary layer is present at the 670-km discontinuity, but this is controversial⁴. Downward movements seem to be dominated by arcuate sheets associated with the subduction of the lithospheric plates observed near trenches and island arcs. In contrast, hot mantle upwellings appear columnar and plume-like. Mid-ocean ridges, which migrate relative to the plumes as a result of the global integration of forces acting on plates⁵, seem to be passive features tapping merely upper-mantle material in response to plate diver-

gence. On the other hand, rising mantle plumes, such as beneath Hawaii or Iceland, more directly reflect the convective state of the deeper mantle. This rough picture is based on surface observables such as plate tectonics, heat flow, the geoid and topographic swells and lineaments⁶⁻⁸, and three-dimensional constraints from seismic tomography⁹ and from numerical and laboratory studies of fluid dynamics³.

Here I focus on the interaction and the dispersal of plumes in the upper mantle, using the spatial distribution of isotope and trace element ratios in basalts from mid-ocean ridges and ocean islands as tracers of mantle dynamics^{10,11}, much as dyes are used to follow flow patterns and mixing conditions in laboratory studies of fluid dynamics. This is possible because mantle plumes are isotopically distinct from the asthenosphere which normally feeds the mid-ocean ridges^{11,12}. This tracer approach, combined with topographic anomalies, provides a means of constraining the volumetric flux of plumes and their excess temperatures relative to the asthenosphere.

On this basis, I evaluate the effect of migrating ridges on the dispersal of mantle plumes. Plumes now as much as 1,000 km from a ridge seem to continue to discharge practically entirely into migrating ridges, which act as sinks. Of the 13 plumes investigated (Fig. 1), the discharge rates range from 0.4 to 3 km³ yr⁻¹. Remarkably, the excess temperatures of these plumes fall in the narrow range of 160-280 K, and their variations are apparently independent of their volumetric flux. For

comparison, the flux of the purely intraplate Hawaiian plume is $9\text{--}12\text{ km}^3\text{ yr}^{-1}$, and its excess temperature is $225\text{--}300\text{ K}$ (refs 7,13). The excess temperatures of these plumes are also independent of their distance to the migrating ridge involved and of their compositional diversity as reflected by their isotope ratios of helium, lead, neodymium and strontium. These surprising results suggest that a quasi-steady thermal state is rapidly established between the thermal plume, the asthenosphere and the accreting and aging lithosphere from the time these plumes were ridge-centred. They also indicate a common, heat-regulated, source in the mantle, which I suggest is likely to be the D" thermal boundary layer at the core-mantle interface.

Plume dispersal models

Several plume dispersal models are currently being debated. For instance, White and McKenzie¹⁴ consider that plumes disperse horizontally beneath the rigid lithosphere in an essentially radial fashion over distances of $1,000\text{--}2,000\text{ km}$. By contrast, Sleep¹³ considers that drag from the plate skews the dispersal of intraplate plumes injected into the low-viscosity zone beneath the lithosphere.

The blob-cluster model suggests that the breakup and dispersal of mantle plumes is directly proportional to the convective rate of the upper mantle, as measured by the spreading rate of mid-ocean ridges¹⁵. It implies a direct link between mantle convection and relative plate motions, and an inverse relationship between spreading rate and variance in isotopic composition in MORBs (mid-ocean-ridge basalts).

Plumes tilted markedly in the upper mantle by plate drags, or by deeper return flows associated with the subduction of plates, may enhance entrainment of the surrounding mantle and detachments of blobs once a critical tilt angle is reached¹⁶⁻¹⁸.

I have pursued the mantle-plume source/migrating-ridge sink (MPS/MRS) model, which suggests that the migrating ridge is fed and dynamically affected by a preferential plume flow along a thermally induced channel at the base of the lithosphere. The inverted channel is progressively carved by the hot plume from the time it was ridge-centred^{10,11,19} (Fig. 2). When ridge-centred, the preferential plume flow takes place along the ridge axis, roughly symmetrically about the plume^{10,20,21}. Mixing of the

rising plume with the asthenosphere takes place by entrainment and in the melting zone, which is initiated at greater depth than along normal ridge segments because the plume is hotter. A larger mean degree of melting and a thicker crust result. A key feature is that if the model¹⁰ is to generate the excess topography and the geochemical gradients observed, the plume flux must exceed the production rate of new lithosphere integrated along the ridge across the plume diameter (on-axis case, Fig. 2a), or over the width of the lateral plume channel at its intersection with the migrating ridge axis (off-axis case, Fig. 2b).

It is now well documented that mid-ocean ridges migrating away from hot mantle plumes are geochemically and dynamically affected by plumes discharging over long times (t) and ridge migration distances (x). The flow connection between plumes and migrating ridges has been established from the following surface observables: tracks of constructional volcanism¹⁹, the geochemical anomaly length, W , along the ridge and the associated excess ridge elevation¹⁰, ΔE (Fig. 2), and the identification of plumes from mixing vectors in multiple-isotope-space representations of basalt compositions^{12,22}.

The empirical relationships $W = 1,010 - 24.5x^{1/2}$ and $\Delta E = 2.42 \times 10^{-3} W$ (with W and ΔE in km), previously established¹⁰ for nine plumes from the Atlantic and Pacific, suggest that these plumes would become purely intraplate when the migrating ridge distance is $\sim 1,700 \pm 250\text{ km}$. At this point, both the topographic and the geochemical anomalies at the migrating ridge axis have disappeared (that is, subsided and contracted to zero). Additional arguments supporting this model of channelled plume dispersal are given elsewhere^{10,22}.

Plume fluxes

Using the above tracer and topographic information, and the concept of buoyancy flux developed for intraplate plumes^{7,13}, I have developed two independent methods for constraining plumes fluxes, or plume discharge rates into migrating ridges.

Method 1 uses for constraint the length (W) of the geochemical anomaly and the nature of the geochemical gradient observed along the migrating ridge axis, for both the on-axis and the off-axis plume configurations shown in Fig. 2. Assuming symmetric conditions about the point of plume injection at $y = 0$,

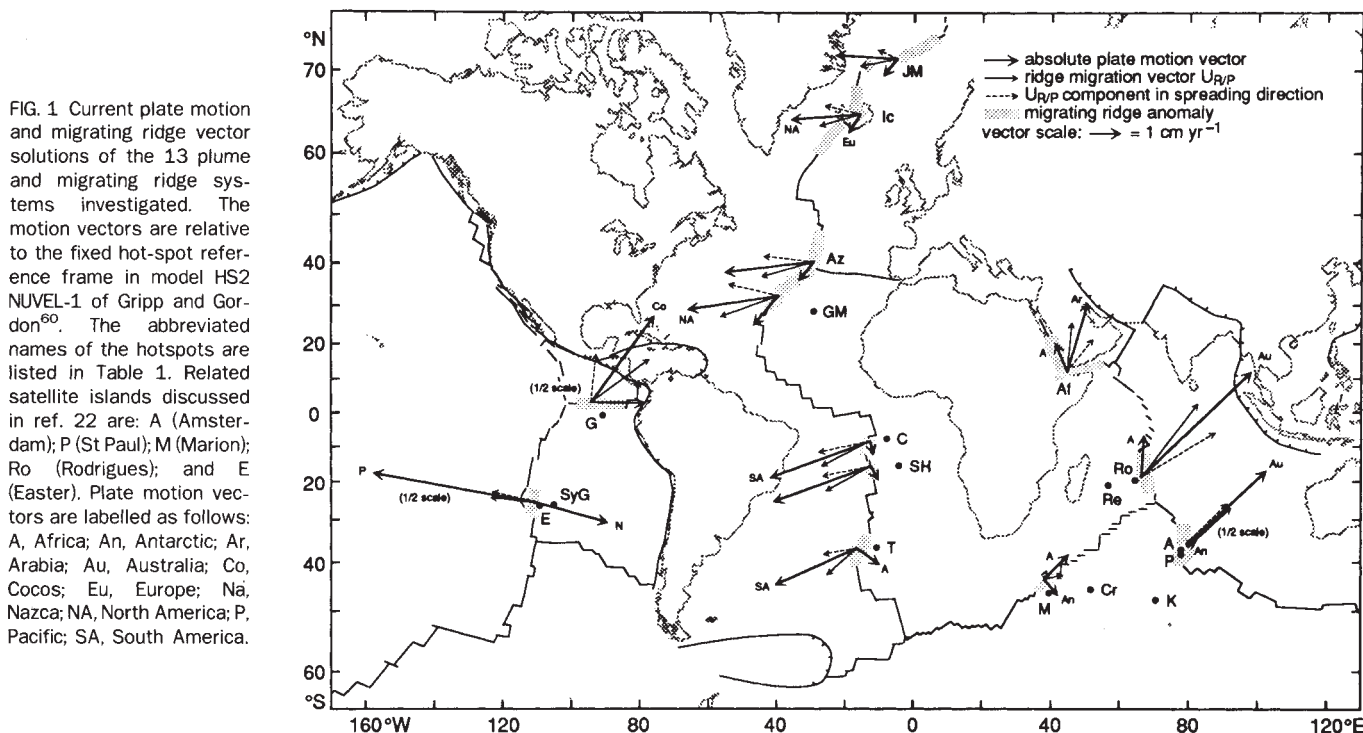


FIG. 1 Current plate motion and migrating ridge vector solutions of the 13 plume and migrating ridge systems investigated. The motion vectors are relative to the fixed hot-spot reference frame in model HS2 NUVEL-1 of Gripp and Gordon⁶⁰. The abbreviated names of the hotspots are listed in Table 1. Related satellite islands discussed in ref. 22 are: A (Amsterdam); P (St Paul); M (Marion); Ro (Rodrigues); and E (Easter). Plate motion vectors are labelled as follows: A, Africa; An, Antarctic; Ar, Arabia; Au, Australia; Co, Cocos; Eu, Europe; Na, Nazca; NA, North America; P, Pacific; SA, South America.

and a steady state between the plume source and the ridge sink¹⁰, the volumetric plume flux Q_p is given by:

$$Q_p = 2k_1 \int_0^L H(y)S(y)P(y) dy = k_1 HSW/2 \quad (1)$$

where $S(y)$ is the full spreading rate, $H(y)$ is the thickness of the fully developed lithosphere and k_1 is a coefficient described below. For a first-order integration, S and H were assumed constant and independent of y . Equation 1 also assumes for simplicity that the fraction, $P(y)$, of the plume contributing to the accretion process per unit ridge length, monitored by the isotopic gradient, decreases symmetrically and linearly with distance, y , along the ridge axis from the centre of the plume, that is $P(y) = 1 - (y/L)$ (Fig. 2)¹⁰.

For a ridge-centred plume (Fig. 2a), Q_p is the total flux of the plume, whereas for the off-axis case (Fig. 2b) Q_p represents only that portion of the plume that is channelled to the ridge axis. The coefficient k_1 then takes into account the fraction of the plume $[1 - (1/k_1)]$ that does not enter the accretion process and may be dragged by the plates and dispersed into the asthenosphere on either side of the ridge axis in the direction $\pm x$ (Fig. 2). The inverse of k_1 ($0 \leq 1/k_1 \leq 1$) is the fraction of the plume flux that is participating in the lithosphere accretion over the anomaly length W (which is twice the integral in equation 1 divided by Q_p). Thus, k_1 must be greater than 1 and depends on the geometry of the kinematic model considered for the dispersal of the plume beneath the lithosphere.

Method 2 uses for constraints the excess elevation ΔE , of the ridge axis associated with the geochemical anomaly (Fig 2), and the concept of the buoyancy flux^{7,13}, adapted to a ridge axis. For both the on-axis and off-axis plume configurations shown in Fig. 2, the mass production rate of excess ridge topography, B , assuming symmetry about the point source of plume injection at $y = 0$, is given by:

$$B = 2k_2(\rho_m - \rho_w) \int_0^L \Delta E(y)S(y) dy = k_2 \Delta E(o)SW(\rho_m - \rho_w)/2$$

The first-order integration was carried out by assuming that $S(y)$ is independent of y . It also assumes a linear gradient for the excess elevation of the ridge axis (ΔE), symmetrically distributed about the centre of the plume (denoted o), that is $\Delta E(y) = \Delta E(o)(1 - (y/L))$ (Fig. 2). The parameters ρ_m and ρ_w are the densities of the mantle ($3,300 \text{ kg m}^{-3}$) and of water ($1,000 \text{ kg m}^{-3}$). The coefficient $k_2 = (\Delta E(o) - \epsilon)/\Delta E(o)$, with $0 \leq k_2 \leq 1$, corrects for the possible contribution of a thicker crust to the ridge elevation anomaly. The parameter ϵ is the contribution to the elevation anomaly $\Delta E(o)$ observed over the point of plume injection, resulting from the thicker crust. It can be estimated from appropriate Airy compensation models, as discussed later.

If the buoyancy of the plume (per unit of gravitational acceleration) is purely of thermal origin, as assumed by Sleep¹³, B is also equal to:

$$B = k_2 \Delta E(o)SW(\rho_m - \rho_w)/2 = \Delta \rho_p Q_p = \rho_m \alpha \Delta T Q_p$$

where the parameter $\Delta \rho_p$ is the density reduction of the thermal plume relative to the ambient mantle, ΔT is the excess temperature of the plume relative to the asthenosphere and α is the thermal expansivity ($3 \times 10^{-5} \text{ K}^{-1}$). Solving for the volumetric plus flux Q_p gives:

$$Q_p = [k_2 \Delta E(o)SW(\rho_m - \rho_w)]/[2\rho_m \alpha \Delta T] \quad (2)$$

The volumetric flux of the 13 plumes investigated by these two methods are compared in Table 1 and Fig. 3a, assuming first $k_1 = k_2 = 1$, $H = 100 \text{ km}$ and $\Delta T = 225 \text{ K}$. The choice of conditions facilitate comparison with Sleep's estimates of plume flux, monitored directly over the plume in off-axis or on-axis settings.

The validity of assuming that the excess temperature is constant will be examined in the next section.

The fluxes estimated by methods 1 and 2 correlate well for plumes at distances $x > 275 \text{ km}$ from the migrating ridges under scrutiny (Fig. 3a). Accordingly, we can infer that in these off-ridge cases, $k_1 = k_2 = 1$, and the plume discharge rates measured at these migrating ridges represent essentially the entire plume component reaching the migrating ridge. There is no detectable excess component of plume flow caused by plate drag, and the crust thickness is essentially normal. The length of the geochemical anomaly, W , reflects essentially the width of the lateral plume channel at its intersection with the ridge axis (Fig. 2b). The local variability of isotope ratios within the anomaly, which is usually greater in these off-axis plume settings^{10,11}, reflects incomplete mixing of entrained asthenospheric material

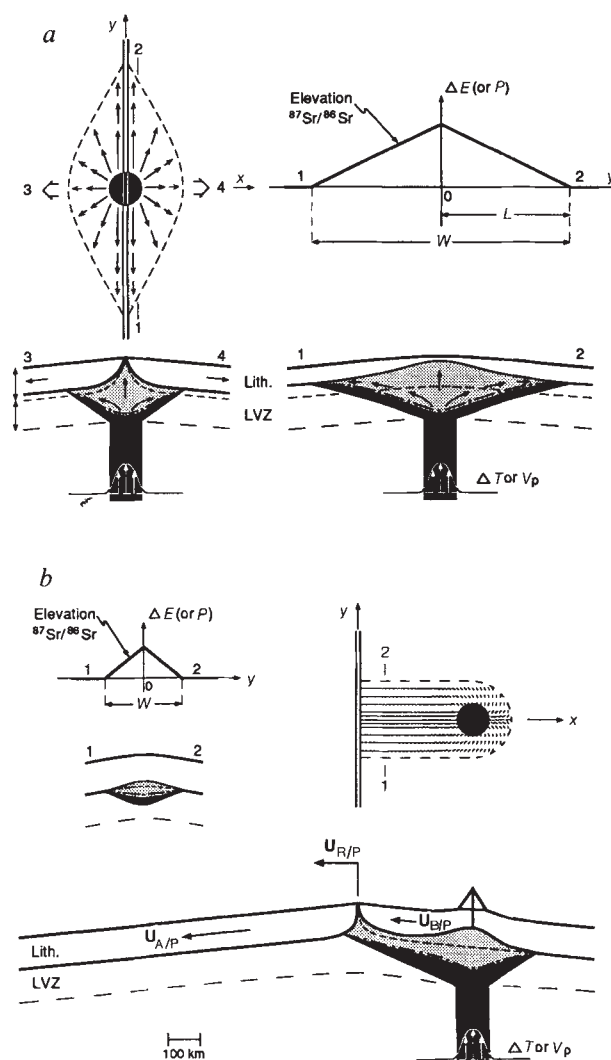


FIG. 2 Sketch of the MPS-MRS kinematic model in plan and section views, shown at two stages of evolution. *a*, Ridge-centred plume position; *b*, off-ridge plume position. Black regions represent the plume, shaded regions represent the zone of partial melting due to decompression. The dashed-dotted line is the solidus; the small dashed line is the base of normal lithosphere outside the thermally induced horizontal plume channel. The resulting excess elevation and strontium isotope gradients along the ridge axis are assumed to be linear in the model. Sections 1 and 2 are parallel, and sections 3 and 4 perpendicular to the ridge axis. $U_{R/P}$, $U_{A/P}$ and $U_{B/P}$ are the horizontal velocity vectors of the migrating ridge axis (R), the plate (A) and the plate (B), relative to the vertical plume (P). V_p is the vertical velocity of the plume relative to the ambient mantle. The LVZ ('low-viscosity zone') is a layer of low seismic velocity underneath the lithosphere. See text for definition of other terms.

in the lateral channel (Fig. 2b). In contrast, in those settings where the plume is located close to or over the migrating ridge ($x < 275$ km), the coefficients k_1 and k_2 must be modelled.

The fraction of the plume flux $[1 - (1/k_1)]$ that disperses into the low-viscosity zone (LVZ) without entering the accretion process can be estimated from the kinematic model proposed by Sleep¹³ for the Iceland ridge-centred plume. Its flux is given by $Q_p = S(H + A/2)W$, where A is the thickness of the LVZ. The model assumes that the component of the plume flow perpendicular to and symmetric about the ridge axis decreases linearly to zero with increasing depth across the LVZ because of plate drag (Fig. 2a). Combining this with equation 1 gives $k_1 = 2 + (A/H)$. If $A = H$, as assumed by Sleep¹³, $k_1 = 3$ in this particular case. I believe that this is likely to be an overestimate of k_1 for two reasons. First, Sleep's model apparently assumes that the accreting lithosphere over the geochemical anomaly length W is composed entirely of plume material, whereas here I assume a linear geochemical gradient (see equation 1 and Fig. 2a) and thus that only half the lithosphere consists of plume material. Second, Sleep's model assumes that the component of the plume flux per unit ridge length perpendicular to the ridge axis, which is being dragged by the plates and dispersed into the LVZ, is constant along the entire length W . Again this is not compatible with the observed geochemical gradient, which suggests that the plume flow decreases outward along the ridge.

A more realistic kinematic plume model is to assume that the plume component per unit of ridge length that is dragged by the lithosphere on either side of the ridge axis decreases in the same linear proportion, $P(y) = 1 - (y/L)$, as observed within the lithosphere, where the decrease is deduced from the geochemical tracers (Fig. 2a). It can easily be shown that this kinematic model corresponds to a plume flux $Q_p = S(H/2 + A/4)W$. Assuming again that $A = H$ for ease of comparison with Sleep's plume fluxes, and combining with equation 1, we obtain $k_1 = 3/2$, which I believe is likely to be an upper limit for this coefficient.

The contribution ε to the observed elevation anomaly $\Delta E(o)$,

resulting from the presence of a thicker crust, can be estimated to first order from the Airy isostatic compensation models shown in Fig. 4. For the Azores submerged platform²³, underlain by an anomalously thick crust given by $H_c = 10$ km, and surrounded by a normal oceanic crust of thickness $h_c = 7$ km (Fig. 4a), $\varepsilon = H_c - h_c (\rho_m - \rho_c) / (\rho_m - \rho_w)$, where ρ_c is the density of the crust ($2,900 \text{ kg m}^{-3}$). In this case ε would be equal to 0.52 km, and accordingly $k_2 = 0.71$. The same value is assumed for the Jan Mayen platform because of the similar setting, as its crustal thickness is not yet documented.

In the case of the Iceland subaerial platform, the Airy model shown in Fig. 4b requires.

$$h_w = [(H_c - h_c)(\rho_m - \rho_c) - h_a \rho_m] / (\rho_m - \rho_w)$$

with, in this case, $\varepsilon = h_a + h_w$, where h_a is the elevation above sea level over the plume, and h_w is the water depth over the normal crust ($y > L$). The anomalous crustal thickness for Iceland is²⁴ $H_c = 16$ km, with a subaerial exposed part $h_a = 0.5$ km, $\varepsilon = 1.35$ km, and $k_2 = 0.6$. If H_c for Iceland were 20 km, as assumed by McKenzie²⁵, then $k_2 = 0.4$, other things being equal. An average of $k_2 = 0.5$ is adopted for Iceland, for the Afar²⁶ and for Crozet²², in view of their similarity in crustal thickness and structure²⁵. The anomalous Galapagos spreading centre probably has a thicker crust than normal, requiring an Airy correction similar to the Azores ($k_2 = 0.7$), but it is sufficiently remote from the plume to assume $k_1 = 1$ ($x = 250$ – 300 km). Overall, it seems that without correcting for the possible presence of a thicker crust, the plume flux would be overestimated by 30–60% at most with method 2.

My 'best estimates' for the 13 plumes so far investigated (Table 1) are based on a simple average of the two methods with $k_1 = k_2 = 1$ for distant plumes ($x > 275$ km), and a weighted average, using the k_1 and k_2 values just discussed, for the ridge-centred plumes (Iceland, Afar and Crozet) and nearly ridge-centred plumes (Azores, Jan Mayen and the Galapagos). The volumetric flux of the 13 plumes investigated ranges from $0.4 \text{ km}^3 \text{ yr}^{-1}$ for the Great Meteor to $3 \text{ km}^3 \text{ yr}^{-1}$ for Kerguelen.

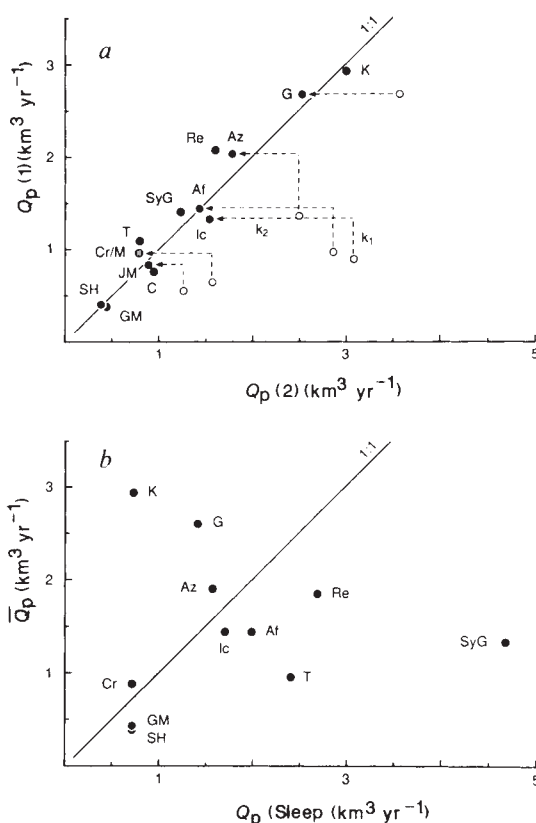


FIG. 3 Comparison of plume discharge rates at the migrating ridge axis Q_p , based on a, the isotope anomaly length (equation 1) and the excess ridge elevation anomaly (equation 2). Dashed lines show the effect of the coefficient k_1 (vertical) and k_2 (horizontal) for ridge-centred plumes ($x < 275$ km), as discussed in the text. b, Best estimate of the plume flux $\bar{Q}_p$ monitored at the migrating ridge axis (average of equations 1 and 2) against the plume flux of Sleep¹³ estimated directly over the plume. The abbreviations for the plume names are given in Table 1.

TABLE 1 Volumetric flux and excess temperature of plumes

Plume	Ridge anomaly	W (km)	$\Delta E(o)^*$ (km)	Dist. (x) (km)	S (mm yr ⁻¹)	$Q_p(1)^\dagger$ (km ³ yr ⁻¹)	k_1	$Q_p(2)^\dagger$ (km ³ yr ⁻¹)	k_2	$\bar{Q}_p^\ddagger$ (km ³ yr ⁻¹)	Q_p (Sleep) (km ³ yr ⁻¹)	ΔT (K)
North Atlantic												
Jan Mayen (JM)	71°–73° N	668	2.2	75	16.6	0.55	1.5	1.26	0.71	0.86	—	242
Iceland (I)	60°–66° N	923	3.4	0	19.0	0.88	1.5	3.08	0.50	1.43	1.98	263
Azores (Az)	33°–40° N	1,094	1.8	100	24.6	1.35	1.5	2.50	0.71	1.90	1.56	198
Great Meteor (GM)	34°–36° N	323	1.1	866	23.8	0.38	1	0.44	1	0.41	0.71	255
South Atlantic												
Circe (C)	7°–11° S	393	1.2	444	38.7	0.76	1	0.94	1	0.85	—	278
St. Helena (SH)	15°–16° S	208	0.9	811	39.3	0.41	1	0.38	1	0.40	0.71	209
Tristan (T)	35°–40° S	565	0.7	416	38.5	1.09	1	0.79	1	0.94	2.4	162
Red Sea/Gulf of Aden												
Afar (Af)	42°–51° E	1,000	2.9	0	19.1	0.96	1.5	2.86	0.50	1.44	1.7	224
Pacific Ocean												
Galapagos (G)	95°–86° W	880	1.3	255	60.2	2.65	1	3.56	0.71	2.60	1.4	214
Sala y Gomez (SyG)	25°–28° S	350	0.85	730	80.0	1.40	1	1.23	1	1.31	4.67	197
Indian Ocean												
Crozet (Cr)	43°–46° S	788	2.38	1,128	16.2	0.64	1.5	1.57	0.50	0.87	0.71	184
Kerguelen (K)	33°–40° S	862	1.0	1,412	67.2	2.90	1	2.99	1	2.94	0.71	232
Reunion (Re)	12°–22° S	892	0.75	1,250	46.3	2.06	1	1.60	1	1.83	2.69	174

Sources of data for Indian Ocean and Sala y Gomez plumes are compiled in ref. 22; for other plumes see ref. 10.

* $\Delta E(o)$ is the maximum excess elevation relative to 2.9 km normal mean depth⁵⁹.

† Given values of $Q_p(1)$ and $Q_p(2)$ are calculated using equations 1 and 2 with $k_1=1$ and $k_2=1$.

‡ Best estimate of plume flux $\bar{Q}_p = (k_1 Q_p(1) + k_2 Q_p(2))/2$ using the values of $Q_p(1)$, $Q_p(2)$, k_1 and k_2 given in the table.

For comparison, the plume flux at Hawaii, the largest currently active flux so far monitored^{7,13}, is 9–12 km³ yr⁻¹. The estimate for Crozet (or Marion) is tentative at best as W and ΔE are inadequately known²².

The fraction of the plume flux radially dispersed into the LVZ (that is, not channelled towards the migrating ridge) can in principle be deduced from the difference between the plume flux estimated by Sleep¹³ from the production of excess topography directly over the plume and the plume discharge rates estimated at the migrating ridge. Sleep's plume fluxes are plotted against the corresponding discharge rates estimated at the migrating ridge in Fig. 3b. The deviation from the mean of my flux and that of Sleep is generally ± 20 –60%, except for Sala y Gomez (Easter), Kerguelen and Tristan where it is ± 90 –120%. The difference are both positive and negative, and show no systematic trends with respect to the ridge migration distance x . Such trends might have been expected, as the lateral plume channel extends and cools with time, and some of the plume material may be dispersed and sink back into the underlying mantle. Some of the larger discrepancies between the two estimates of plume flux, monitored directly over the plume and at the intersection with the migrating ridge, are inherent in the choice of parameters used in the two models and can readily be reduced by normalization. For example, Sleep assumed a half-width ridge anomaly of 400 km comprising both Tristan and Gough hotspots, whereas I have used $L = 250$ km for Gough only. For the Easter region, Sleep used a full spreading rate of 200 mm yr⁻¹ for the East Pacific Rise, whereas I used 80 mm yr⁻¹, as we have shown that only the east rift of the Easter microplate is affected by the Sala y Gomez plume^{27–29}. As for the large negative discrepancy for Kerguelen, which is absurd, Sleep's flux estimate is not specific to this particular plume but generic to weak intraplate plumes at the detection limit of his method. In turn, it may be that my estimate made for the southwest Indian Ridge is related to the St Paul or Amsterdam plume instead of the more remote Kerguelen hotspot, as also evident from the isotope signals²². Overall, it thus seems likely that the discrepancies between Sleep's flux values and those obtained in my approach reflect the large errors involved in the calculations, which can easily reach 50% in my estimates. Nevertheless, the broad similarity of the plume fluxes estimated over the vertical plume and at the migrating ridge suggests that

the discharge at the ridge can represent a substantial fraction of the total plume flux dispersed into the LVZ.

Excess temperature of plumes

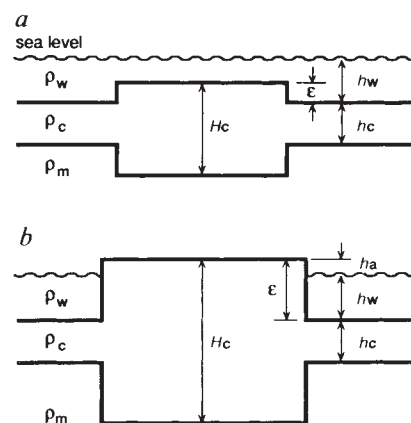
By equating the two plume flux methods 1 and 2 given above, I have solved for the mean excess temperature of plumes, which is given by:

$$\Delta T = [k_2(\rho_m - \rho_w)\Delta E(o)]/[k_1 H \rho_m \alpha] \\ = 232(k_2/k_1)\Delta E(o)$$

with ΔT in K and $\Delta E(o)$ in km, $H = 100$ km and the previously quoted values for ρ_m , ρ_w and α . Again, this assumes that the buoyancy of the plume is purely thermal in origin. The mean temperature excess of the plume is thus directly proportional to the maximum excess elevation on the ridge, within the uncertainty of the factor (k_2/k_1) . Without this correction, ΔT would be inversely related to the distance of the migrating ridge to the plume and range from 800 to 200 K (Fig. 5). We have just seen, however, that (k_2/k_1) may range from 0.33 to 0.47 for ridge-centred plumes. With these corrections, and $k_1 = k_2 = 1$ for the plumes more than 275 km from the ridge, the excess temperatures of the 13 plumes fall in the narrow range of 162 K (for Tristan) to 278 K (for Circe), including 263 K for Iceland, and their average is 215 ± 35 K (1σ) (Table 1 and Fig. 5). The range quoted compares well with other estimates using independent methods and constraints. These vary from 100 K for Iceland²¹ to 300 K for Hawaii³⁰, and are generally 200–250 K (refs. 7,13,25,31). The inversion performed by McNutt³² indicates an excess temperature of 250–300 K at a depth of 50 km beneath Hawaii and the Marquesas. Finally, Sleep¹³ has noted that the centre of these plumes may be 1.25 to 1.6 times as high as the mean excess temperature discussed here (estimated along the ridge axis over the length of the elevation and geochemical anomaly). Surprisingly, the excess temperature of the 13 plumes show no relation to the migrating ridge distance x , nor to their discharge rates at the migrating ridge. This observation has several implications.

First, it suggests that a quasi-steady thermal state is established rapidly at shallow depth, between the plume, the LVZ and the accreting and aging lithosphere, from the time these plumes were centred over the ridge to their present configurations

FIG. 4 Airy isostatic compensation models for ridge-centred plumes. *a*, Submerged platform (such as Azores); *b*, Subaerial platform (such as Iceland). See text for definition of terms.



(Fig. 1). Implicit in this MPS/MRS model is that the plume flow takes place mostly along a thermally induced groove and rounded channel carved by the hot plume at the base of the accreting lithosphere (Fig. 2). Using Turcotte and Oxburgh's thermal boundary-layer model¹ and a typical excess temperature of the plume of 200 K, I estimate²² this thermal groove to be ~30 km deep, measured upward from the base of a fully developed lithosphere of 100 km thickness, and only 3 km deep near the migrating ridge axis where the lithosphere is only 10 km thick. The pressure gradient required to maintain this lateral plume flow toward the migrating ridge is provided by the dynamic pressure of the plume guided by the slope of the growing rigid lithosphere towards the ridge axis. Elsewhere²², I present kinematic arguments that suggest that forces resulting from plate drag or large-scale return flows associated with the plate tectonic cycle have smaller effects. Assuming a Poiseuille type of conduit plume flow, 100 km in diameter and, 1,000 km long, with a $3 \text{ km}^3 \text{ yr}^{-1}$ flux and a viscosity of $3 \times 10^{18} \text{ Pa s}$, the mean pressure drop required would be 116 Pa m^{-1} , corresponding to a hydraulic head of 3.5 km. This is not impossible, as an inviscid plume from the core-mantle boundary³³, with a density contrast of 33 kg m^{-3} , would have a hydraulic head of 29 km.

Second, it is now established that the excess temperature of starting plume heads³⁴, as well as of plume conduits tilted by mantle winds^{18,35}, decreases as they rise and entrain the surrounding mantle. The magnitude of the entrainment depends on the buoyancy (heat) flux of the plume. Weak plumes (small flux) entrain and cool more than strong ones, other things being equal. Thus, for a given ΔT at the source, one would expect the ΔT of the conduit plumes in the upper mantle to be either inversely proportional to their depth of origin for equivalent

fluxes, or directly proportional to their fluxes if they came from the same depth and had the same tilt. The latter conditions are evidently too restrictive, as the plume fluxes do not correlate with their excess temperatures in the upper mantle.

We also observe no apparent correlation between the excess temperature and the geochemical and isotopic composition of the plumes, as one might expect if some of the plumes under investigation came from the core-mantle boundary³⁶⁻³⁸ and others from the 670-km discontinuity³⁹, or if some had been triggered at shallower depth by some kind of metasomatism⁴⁰. For example, the high ratio $^3\text{He}/^4\text{He}$ in plumes such as Iceland is usually taken to indicate a derivation from the lower mantle⁴¹, whereas low $^3\text{He}/^4\text{He}$ in plumes such as the Azores may reflect crustal recycling at shallower depths⁴². Yet the range of ΔT of plumes with high $^3\text{He}/^4\text{He}$ is 263 K (Iceland)⁴¹ to 174 K (Reunion)⁴³, and for plumes with low $^3\text{He}/^4\text{He}$ it is 232 K (Kerguelen)⁴⁴ to 162 K (Tristan)⁴⁵. The overlap is essentially complete.

A derivation from the lower mantle has also been suggested for plumes with the Dupal isotopic signature as a result of the coincidence of the Dupal anomaly with the second-harmonic residual geoid anomaly and P-wave velocity anomaly determined by seismic tomography⁴⁶⁻⁴⁸. But again there is no clear systematic relationship between ΔT and the isotopic characteristics. The ΔT of the Dupal plumes investigated from the South Atlantic and the Indian oceans is 162-232 K, whereas for the non-Dupal plumes $\Delta T = 170-263 \text{ K}$. Again the overlap is complete. Thus, it also seems that the contents of the heat-generating elements uranium, thorium and potassium are not likely to be influential internal factors in controlling the excess temperature of these plumes. This has also been pointed out by Griffiths⁴⁹ on the basis of fluid-dynamic arguments.

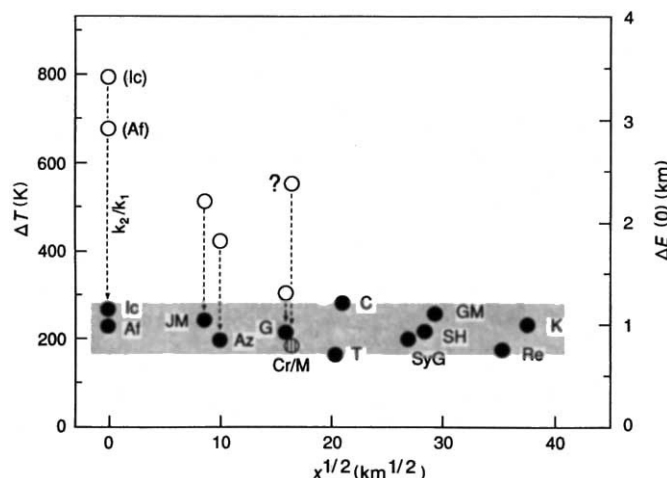


FIG. 5 Plume excess temperature plotted against the square-root of the distance to the migrating ridge. The square-root distance scale is used for visual convenience only. Vertical dashed lines show the effect of the coefficient k_2/k_1 for the ridge-centred plumes ($x < 275 \text{ km}$) ○, uncorrected data for $x < 275 \text{ km}$; ● for $x > 275 \text{ km}$, or as given in Table 1 and discussed in the text. Note that the estimated excess temperatures of the plumes fall in the narrow range of 160-280 K (shaded region). The variation in ΔT is independent of the distance x to the migrating ridge, the volumetric flux and the He, Pb, Nd and Sr isotope composition of these plumes. The abbreviations for the plume names are those given in Table 1.

At this stage and within the uncertainties in ΔT s involved, which certainly are ± 50 K, and could possibly reach 100 K, it would seem that a common depth of origin and regulated source of heat is indicated by the relative constancy of the estimated excess temperatures of the 13 plumes investigated. The D'' thermal boundary layer at the core-mantle interface is the most likely place³⁶⁻³⁸. If so, then what caused the large diversity in isotope and trace element ratios among these plumes? Perhaps the geochemical and isotopic diversity of plumes, and their temporal variation so far documented, reflect the layered nature and timing of the recycled lithosphere material accumulating and forming the D'' layer, the mode of subsequent plume detachments and the complexities of the entrainment process, particularly across the 670-km discontinuity where some recycled material may also have accumulated^{39,50,51}.

It is of interest to note that Bonatti⁵² has described the mantle underlying the Azores as "not so hot" on the basis of geothermometry of peridotites from the region. The Azores is a low ³He/⁴He 'wet' plume highly enriched in volatiles⁴⁰. My estimate of its excess temperature, however, is 198 K, well within the range of other plumes. Therefore, this claim cannot be confirmed, unless it could be demonstrated that in this case the buoyancy flux of the plume is of chemical rather than of thermal origin, but this is doubtful.

Estimates of plume excess temperatures by my method could be improved in the future with new information on crustal thicknesses associated with these anomalous migrating ridge segments, with improved topographic maps and sampling, and by adding constraints from geoid data. Distinguishing between plume origins from estimates of excess plume temperatures is likely to be a difficult task, however, because of the uncertainties involved in the method and in the effect of entrainment on the excess temperature in the plume conduit as a function of depth, which remain poorly constrained and quantified.

Other dynamic effects

Finally, there is ample evidence that plumes feeding migrating ridges can have other important dynamic and tectonic effects, apart from topographic uplift. They can generate the propaga-

tion of rifts⁵³ and development of transient microplates²⁷, and induce rift jumps back towards the plume, and as a result, large transform-fault offsets⁵⁴. Variations in isotope tracer concentrations across these transform-fault discontinuities testify that conduit plumes do not disperse radially into the LVZ, but are preferentially channelled as the MPS/MRS model predicts^{10,53}. Apparently, the only time that plumes may disperse radially into the upper mantle may be when an initial plume head reaches and flattens against a rather stationary plate, such as a subcontinental lithosphere. Evidence for a vestige of the dispersal of St Helena and Tristan plume heads under the Gondwana continental lithosphere has been detected¹² along the Mid-Atlantic Ridge between 20° S and 40° S. Also, variations in the isotope tracers neodymium, strontium and lead along the Gulf of Aden and Red Sea suggest that the head of the Afar plume may have recently been flattened and radially dispersed⁵⁵.

The bending of plumes into the LVZ towards migrating ridges, and their temperature structure, also have important effects on the outgassing of plumes and the local decoupling of the ³He/⁴He ratio from the isotope tracers, as well as the petrology of melting products directly over the plume, along the channel and at the migrating ridge axis. These topics are considered elsewhere²², in conjunction with the study of the tilting of some of these plumes by mantle winds^{18,56}, as a possible alternative to the MPS/MRS model discussed here. But that study indicated that tilting by mantle winds was insufficient to explain the observations and the MPS/MRS model is still preferred as the dominant mechanism affecting the topography and geochemistry of mid-ocean ridges migrating away from plumes.

Geophysicists have yet to find a ridge that is currently migrating towards a fixed plume, and that has an isotope signature clearly distinguishable from the depleted asthenosphere usually feeding mid-ocean ridges; such a situation would allow the dynamics of plume-ridge interaction to be studied further. This was thought to be the case for the Juan de Fuca Ridge with respect to the Heck-Heckle-Springfield seamount chain⁵⁷, but lava from these seamounts has mainly been found to be normal MORB-like and different from those found on the nearby Endeavor ridge segment⁵⁸. The search continues. □

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Multiple modes of *engrailed* regulation in the progression towards cell fate determination

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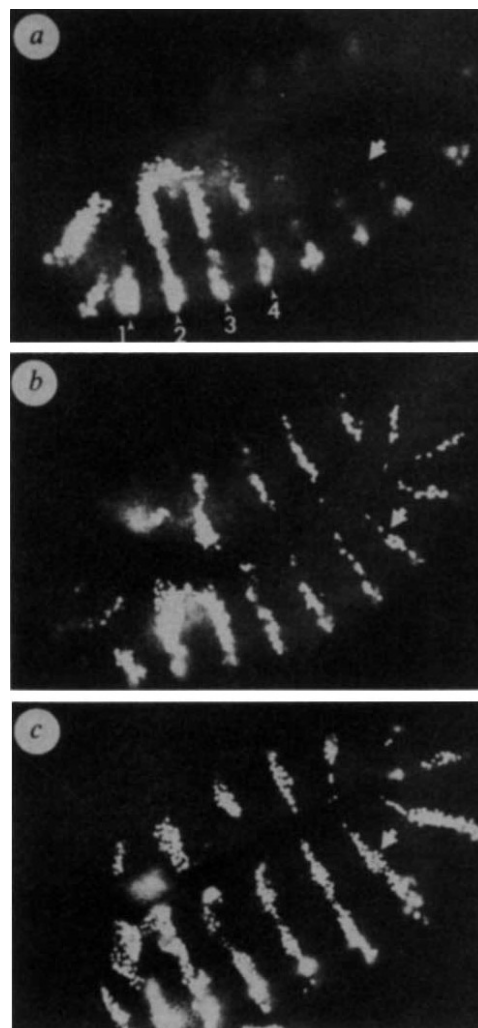
The *engrailed* gene product of *Drosophila* specifies the fate of a subset of cells in each segment. Our studies of *engrailed* regulation suggest that fate determination is an elaborate, multistep process. At the time in embryogenesis when the *engrailed*-dependent cell fate is probably determined, four modes of control act in an overlapping progression to govern *engrailed* expression. After activation by *pair-rule* genes, both an extracellular signal, *wingless*, and autoregulation are required for *engrailed* expression. Autoregulation graduates to *wingless* independence, but is transient, and is superseded by an *engrailed*-independent mode of maintenance.

Two levels of commitment have been defined for the choice of cell fate¹. Early in development, fate can be instructed by environmental cues. This fate specification is flexible and allows fate to change in response to extracellular signals. But cells can undergo a transition from this type of specification to one which is determined and therefore irreversible. Because a determined cell maintains a particular fate independent of its environment, the transition to a determined state requires that development comes under cell-intrinsic, rather than cell-extrinsic control. The identification of selector genes in *Drosophila* provides an opportunity to study the basis of both types of cell-fate control. Selector genes encode regulators whose function defines the fate of a cell, and whose continued expression stably maintains cell fate^{2,3}. Where the choice of cell fate is initially dependent on the environment, selector gene expression must be sensitive to extracellular signals. By contrast, once cell fate is determined,

expression of these genes must be governed exclusively by cell-intrinsic factors.

The *engrailed* (*en*) gene is a selector gene that distinguishes two populations of cells in each developing embryonic segment, the progenitors of the anterior and posterior compartments^{3,4}. This distinction can be seen as a lineage restriction when individual embryonic cells are marked by mitotic recombination^{5,6}. These give rise to clonal patches in the adult that never span the boundary between the anterior and posterior compartments, demonstrating that, from the time of marking, the progeny of a given cell only contribute to one compartment. This restriction requires expression of *en* by posterior cells^{3,4,7}. But it seems that the onset of localized *en* expression in early embryos is not sufficient to irreversibly determine posterior compartment identity because *en* requires at least one cell-extrinsic input at this time.

FIG. 1 Expression of *en* becomes *wg*-independent after 5 h. Embryos are stained with an anti-*en* antibody¹⁴. Magnification 140×, anterior is left and ventral is down in all figures except when noted. Embryos are shifted from 18 °C to 29 °C (at different times, but age of embryos is corrected for effect of temperature and is given in hours after egg laying (AEL) as if embryos were raised at 25 °C. *a*, *wg*^{IL114ts}/*wg*^{CX4} embryo grown at nonpermissive temperature (29 °C) from ~3.5 to 7.5 h AEL. Expression of *en* is indistinguishable from that seen in a *wg* null embryo at this stage^{11,12}. Only expression that is not under *wg* control persists: in the head, the first four ectodermal stripes (1–4), and a segmentally repeated subset of CNS cells. Expression has disappeared from the lateral ectoderm posterior to the fourth *en* stripe (for example, the normal position of stripe 7 is indicated by an arrow). In wild-type embryos at this stage, *en* striped expression is easily detectable (see Fig. 2b). *b*, *wg*^{IL114ts}/*wg*^{CX4} embryo grown at nonpermissive temperature from ~4.5 to 8 h AEL. Some ectodermal cells continue to express *en*, creating a partial stripe in each segment (for example, stripe 7 is indicated by an arrow). *c*, *wg*^{IL114ts}/*wg*^{CX4} embryo grown at nonpermissive temperature from ~5 to 8 h AEL. The *en* stripes are almost wild-type (for example, stripe 7 is indicated by an arrow). Some stripes are incomplete dorsally. This is the same phenotype seen when these embryos are grown exclusively at permissive temperature (18 °C). Immunocytochemistry was done as in refs 14, 18. The *wg*^{IL114ts} is described in ref. 13 and was obtained from the Tübingen stock collection. The *wg*^{CX4} is described in ref. 8.



This input is provided by the *wingless* (*wg*) gene, expressed in segmentally repeated stripes of cells that lie next to *en*-expressing cells^{8,9}. The *wg* protein, which is homologous to the product of the proto-oncogene *Wnt-1* (ref. 10), is secreted and appears to be taken up by *en*-expressing cells⁹. In *wg* mutant embryos, *en* expression is initiated in a normal striped pattern but soon stops, indicating that continued *en* expression in wild-type embryos requires *wg* in neighbouring cells^{11,12}. Here we show that *en* expression becomes independent of this extracellular influence and comes to rely on positive autoregulation. But autoregulation is transient and so does not supply a mechanism for determination of the *en*-cell fate. We show that autoregulation is followed by a still-later-acting mode of *en* control. These transitions may be the progression from a stage in which cell fate is influenced by interactions with neighbours to a later stage in which cells are stably determined.

Transition to *wg* independence

If anterior and posterior compartments become determined, then the selector gene *en* must become independent of the extracellular *wg* influence. Previous experiments with a temperature-sensitive(*ts*) *wg* allele demonstrated that, during embryogenesis, there are changes in the requirement for *wg* in body patterning¹³. To test whether *wg* becomes dispensable for *en* expression, *wg^{ts}* embryos were shifted from permissive to restrictive temperature at half-hour intervals beginning at cellular blastoderm (2.5 h). After further development at restrictive temperature, the embryos were fixed and stained with anti-*en* antibodies (Fig. 1). When *wg^{ts}* embryos are shifted to nonpermissive temperature before 4 h of development, *en* expression decays prematurely in the thoracic and abdominal ectoderm (compare mutant expression in Fig. 1a with wild type in Fig. 2b). Loss of *en* parallels that seen in *wg* null mutants^{11,12}. (We do not consider the regions of the embryo where *en* expression does not involve *wg* control: the head, the first three *en* stripes and the central nervous system (CNS) in each segment.) Between 4 and 5 h, inactivating *wg* has progressively less effect on *en* expression. For example, when *wg* is inactivated at about 4.5 h, a few cells in each segment persist in expressing *en* (Fig. 1b). After 5 h, removing *wg* function has no apparent effect on *en* expression (Fig. 1c). The striped pattern is indistinguishable from that of *wg^{ts}* embryos raised at permissive temperature. We conclude that *en* expression becomes independent of the *wg* signal at about 5 h of development. Thus, different regulators maintain *en* past this time.

Requirement for *en*

Studies of expression of mutant *en* alleles suggest that *en* undergoes autoregulation. The *en^{CXI}* allele is a rearrangement (R. Holmgren, personal communication) that truncates the *en* transcription unit (data not shown) and produces a cytoplasmic protein. For this reason, and because *en^{CXI}* shows a severe *en* phenotype (S.D., unpublished observation), we consider this a nonfunctional protein. We can monitor expression of this allele using an anti-*en* antibody and distinguish it from the nuclear wild-type *en* product because *en^{CXI}* is cytoplasmic. The initial activation of *en^{CXI}* expression occurs normally (compare Fig 2a with c). But in embryos homozygous for *en^{CXI}*, striped expression in the ectoderm stops during full germ-band extension (Fig. 2d), a time when *en* is easily detectable in wild-type embryos^{7,14} (Fig. 2b). Thus, the normal persistence of *en* expression past this stage is directly or indirectly dependent on the action of *en* itself.

A second mutant *en* allele, *en^{IM}*, has a stop codon in the homeodomain that truncates the protein upstream of the DNA binding motif (Gln at position 497 mutated to the stop codon TAG; J. Little and P.O'F., unpublished data). RNA *in situ* hybridization shows that *en^{IM}* mutant embryos fail to maintain *en* transcripts (data not shown). The behaviour of these mutant alleles demonstrates that maintaining *en* expression requires *en* function.

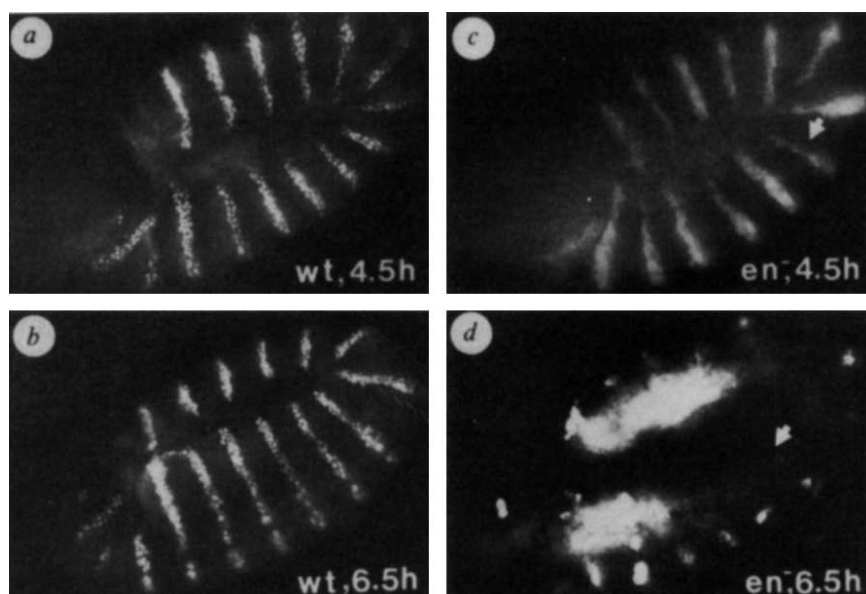
Autoregulation of *en*

To test whether *en* activates its own expression we constructed transgenic flies that carry the *en* gene driven by a heat-shock promoter (Fig. 3a). Embryos that carry this construct (*hs-en*) produce *en* transiently in all cells after a brief heat shock (Fig. 3b). We examined the effect of this protein in cells that do not normally express *en*. Inducing global *en* expression by heat shock had a different effect early, during the *wg*-dependent period for *en*, than it did later, at *wg*-independent stages.

Our standard early heat-shock protocol consists of heat-shocking embryos at 3–3.5 h of development. On recovery, the globally induced *en* expression resolves into stripes that are abnormally broad as a consequence of new *en* expression in the ventral and lateral regions (compare Fig. 3c and d). This new expression pattern is stable and persists to at least 12 h of development when cells begin to differentiate (data not shown).

To verify that the novel *en* pattern results from activation of the endogenous *en* gene, we used *en^{CXI}* as a marker for activity of the *en* promoter. Embryos with one copy of *en^{CXI}* and one

FIG. 2 Expression of *en* stops prematurely in *en* mutants. Embryos (120×) are stained with an anti-*en* antibody. a, Wild-type embryo, ~4.5 h AEL. b, Wild-type embryo, ~6.5 h AEL. c, An *en^{CXI}/en^{CXI}* embryo ~4.5 h AEL. The mutant cytoplasmic protein is expressed in a wild-type pattern. d, An *en^{CXI}/en^{CXI}* embryo, ~6.5 h AEL. The mutant protein has disappeared from the ectoderm at this stage. For example, the normal position of stripe 7 is indicated by an arrow (compare with c). Some cells of the CNS in each segment continue to express the *en^{CXI}* allele. The diffuse signal in other areas of the embryo is yolk autofluorescence. Loss of *en^{CXI}* is not the result of defects in the *cis*-acting control region because this allele is expressed normally when heterozygous with wild-type *en* (data not shown). The *en^{CXI}* stock was obtained from R. Holmgren.



copy of wild-type *en* are phenotypically wild type except for the cytoplasmic accumulation of truncated *en*^{CXI} protein (compare insets in Fig. 3c and e). When heat-shocked, *en*^{CXI} heterozygotes that carry a *hs-en* element express the cytoplasmic *en* protein ectopically, resulting in broadened stripes (Fig. 3e). Therefore, the pulse of *en* produced from the heat-shock promoter results in transactivation of the *en* promoter.

If stable *en* expression after heat shock results from activation of an autoregulatory circuit, then maintaining ectopic *en* expression should require a functional endogenous *en* gene. Indeed, in *en*^{CXI} homozygous mutant embryos the early heat-shock protocol does not give stable ectopic expression. The *en*^{CXI} mutants with or without the *hs-en* insert are nearly indistinguishable, as both lose ectodermal *en* expression (not shown). Thus, maintenance of expression from the endogenous *en* promoter, once activated in new cells by heat-shock-produced *en*, requires the activity of the endogenous *en* gene. We conclude that *en* is subject to positive autoregulation. Because *en* is a transcription factor¹⁵, one simple model for autoregulation is that *en* directly acts at its own promoter. But autoregulation might be indirect and could even involve cell-extrinsic signalling.

Another consequence of heat shock is repression of *wg*. Although the *wg* RNA pattern is almost normal immediately after heat shock (data not shown), on 1 h of recovery from heat shock, *wg* expression is drastically reduced (compare Fig. 4a and b). At this time, *en* is expressed in the broadened stripes

shown in Fig. 3c. The stripes of *wg* RNA have completely disappeared by 2 h after heat shock (Fig. 4c). The *wg* decay is still consistent with its requirement for *en* expression; *wg* RNA is detectable until the end of the *wg*-dependent period as defined with *wg*^{ts}.

The loss of *wg* after heat shock requires the presence of *hs-en*, thus it appears that *wg* is repressed by either the pulse of *en* from the heat-shock promoter or ectopic expression of the endogenous *en* gene. The repression of *wg* by *en* when the two genes are expressed in the same cell may have a function in normal development. This mechanism could assure that the expression of these two genes is established in immediately adjacent but nonoverlapping stripes.

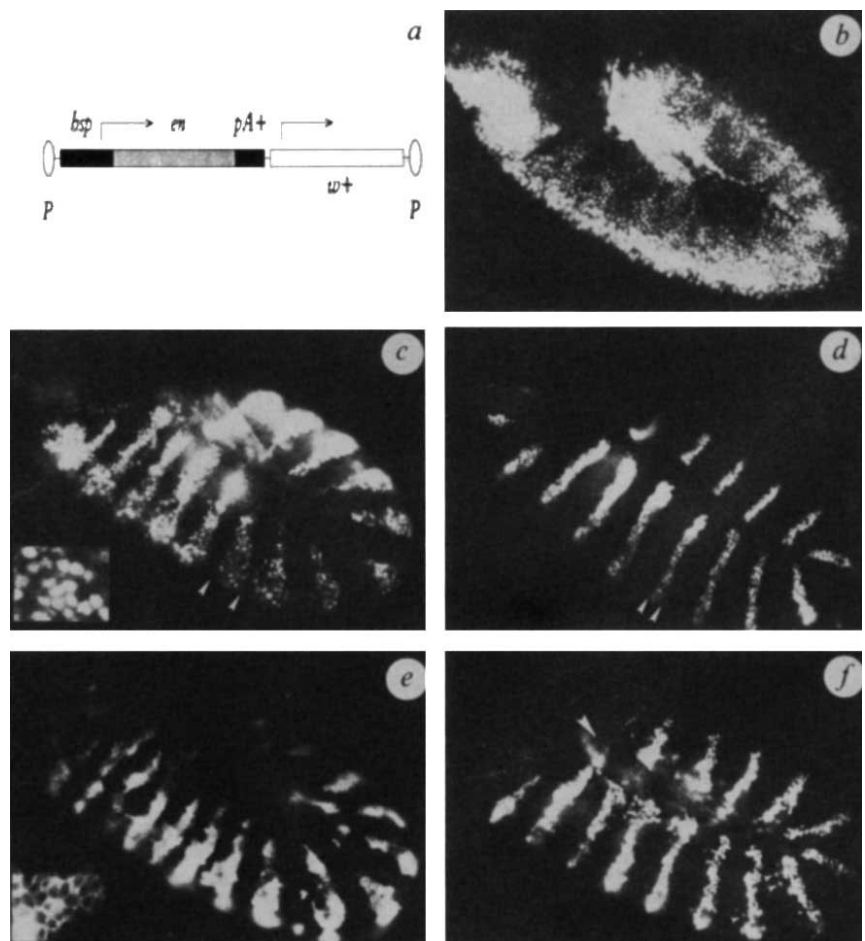
Activation of autoregulation by *wg*

Ectopic *en* expression can only be induced during the *wg*-dependent window (Fig. 3f, described below). We therefore considered the possibility that the initiation of autoregulation requires *wg* input by examining the effect of *hs-en* in *wg*-mutant embryos (*hs-en*; *wg* null). After recovery from standard early heat shocks, *en* expression decays in *hs-en*; *wg* null embryos just as it does in *wg* mutant embryos without *hs-en* (data not shown). Therefore, *wg* function is required for autoregulation at this stage.

As *en* expression normally requires *wg* input transiently in development, this experiment does not exclude the possibility

FIG. 3 Globally induced *en* activates the endogenous *en* gene in novel cells. Embryos (115×) are stained with an anti-*en* antibody. *b–e*, Embryos between 6 and 7 h AEL. *a*, Schematic diagram of *hs-en*. The *en* sequences consist of a 2-kb *EcoRI*-*SnaBI* fragment of the *en* cDNA clone c-2.4 (ref. 32). Transcription of *en* sequences (stippled box) is driven by the *hsp70* heat-shock promoter (*hsp*), contained in a 1.3-kb *SphI*/*PstI* fragment from pUCHSP 70 (provided by E. Gavis). The polyadenylation sequences (*pA+*) are from SV40. For transformation, the above sequences are inserted into the pCaSpeR vector³³ containing P element ends (ovals) and the *white* gene (*w+*) as a selectable marker. *b*, An *hs-en* embryo, heat shocked at ~6 h, then immediately fixed and stained. The *en* expression induced by heat shock is detectable in all cells. *c*, An *hs-en* embryo, heat shocked at ~3 h AEL, aged 3.5 h, then fixed and stained. The *en* stripes are abnormally broad in the ventral and lateral regions compared with wild type (compare distance between arrowheads in *c* and *d*). Inset, 326× magnification of part of one stripe showing that the *en* antigen is confined to the nucleus. *d*, Wild-type embryo, showing the normal pattern of *en* expression. *e*, An *en*^{CXI}/+;*hs-en* embryo, heat shocked at ~3 h AEL, aged 3.5 h, then fixed and stained. The pattern of cytoplasmic *en*^{CXI} antigen is similar to the pattern of *en* in *c*. Inset is a 326× magnification of part of one stripe showing that the cytoplasmic antigen, characteristic of *en*^{CXI}, is produced in novel cells after heat shock. Both the wild-type nuclear and mutant cytoplasmic *en* antigens are expressed in these cells, although the nuclear signal is weak relative to the cytoplasmic signal. *f*, An *hs-en* embryo, heat shocked at ~5 h then fixed after 3 h recovery at 25 °C. Striped *en* staining is nearly wild type. There are isolated cells in the anterior compartment that express *en* (small arrowheads), as well as staining in the amnioserosa (large arrowhead), neither of which is seen in wild type.

METHODS. Transgenic lines were established²⁹ using the p-wings clipped helper and *Df(1)w^{67c2}* as host. For heat-shock experiments, embryos were collected and aged at 25 °C on grape agar plates. At the appropriate stage, plates were put in moist chambers at 37 °C for 35 min. They were then



returned to 25 °C for recovery. The novel expression pattern was detectable as soon as the *en* expressed from the heat-shock promoter decayed to background levels, ~45 min after heat shock. Similar results were seen with three different *hs-en* lines, one on chromosome III and two on chromosome II.

that the *wg* signal is only required before autoregulation is initiated. Therefore, we tested whether the *wg* requirement is concurrent with autoregulation using *wg^{ts}* embryos carrying *hs-en* (*hs-en;wg^{ts}*). Heat shock has two effects on *hs-en;wg^{ts}* embryos raised at permissive temperature: it provides a pulse of *en* to all cells and simultaneously removes *wg* function. We carried out standard early heat shocks in the *wg*-dependent window, then let the embryos develop further at a temperature that is restrictive for the *wg^{ts}* allele. As with heat shock in a *wg* null background, *hs-en;wg^{ts}* embryos failed to give stable ectodermal *en* expression (data not shown). Thus, in early embryogenesis, the cell-extrinsic *wg* signal is required during or after the initiation of autoregulation.

Autoregulation independent of *wg*

Although *en* autoregulation requires *wg* early, normal *en* expression continues after the end of the *wg*-dependent period. To discover whether there is a later stage of autoregulation that is *wg*-independent, we heat-shocked *wg* mutant embryos aged beyond the *wg*-dependent period. Recall that, after about 5 h, *wg* mutant embryos show no *en* expression in the ectoderm at any position posterior to the fourth *en* stripe^{11,12} (Fig. 5a). Surprisingly, in *hs-en;wg* null embryos, a late heat-shock protocol induced stable, albeit variable, *en* expression in the ectoderm (Fig. 5b). Thus, *wg* function is required for autoregulation only early in development; there is a later stage of autoregulation that acts independently of *wg* (Fig. 8a). Perhaps a different positive regulator substitutes for *wg* later, or alternatively, *wg* may counteract an early repressor of *en* that is not present late (Fig. 8b).

The second stage of *en* autoregulation is restricted to fewer cells. The pattern generated in *hs-en;wg* null embryos after late heat shock is different from that seen with earlier heat shocks in wild-type *hs-en* embryos. Although late heat shock induces *en* in a variable number of cells, the cells are distributed in a narrow stripe. These stripes line up with underlying *en*-expressing cells of the CNS, strongly suggesting that these are posterior compartment cells. It appears that, once *en* expression becomes *wg*-independent, only the cells of the posterior compartment are capable of *en* autoregulation (Fig. 8b). We will return to this point below.

Restricting autoregulation

Autoregulation of *en* does not operate in all cells, as globally induced *en* resolves into patterned expression after heat shock (compare Fig. 3b and c). Two possibilities, which are not mutually exclusive, might explain this restriction. First, necessary coactivators may be localized. Second, there may exist repressors that block autoregulation in some cells.

We have identified at least one repressor that restricts autoregulation. In embryos mutant for the segment polarity gene *naked* (*nkd*), *en* is expressed in a broadened stripe pattern¹² (Fig. 6a, compare with Fig. 3d (wt)). Thus, *nkd* directly or indirectly represses *en* in certain cells of the embryo. We tested whether *nkd* activity restricts *en* autoregulation using *nkd* embryos carrying *hs-en* (*hs-en;nkd*). After the standard early heat shock, these embryos stably express *en* in almost all ectodermal cells, except the head and telson (Fig. 6b). This global expression involves more cells than the sum of broadened *en* expression in *nkd* (Fig. 6a) and broadened *en* in *hs-en* (Fig. 3c), at least in the lateral and dorsal regions. Therefore, *nkd* function restricts early *en* autoregulation. But autoregulation is restricted by different factors later in development. Although early heat shocks of *hs-en;nkd* embryos produce global *en* expression, later in development *nkd* mutants are indistinguishable with and without heat shock (data not shown).

The pattern to which *en* autoregulation is restricted after heat shock also changes during development. As shown above, late autoregulation, detectable as reinduction of *en* in *wg* mutants (Fig. 5b), is not activated in cells outside the normal *en* domain.

This is also evident from a comparison of early and late heat shocks in wild-type embryos. Although early heat shocks in wild type induce stable *en* expression in cells outside the normal *en* stripe, heat shock after 4 h does not generate stable ectopic *en* expression, demonstrating a block to autoregulation in cells outside the posterior compartment (Fig. 3f). Using a similar construct, Poole and Kornberg reported the effect of heat-shock-produced *en* on the larval body pattern¹⁶. Only early heat shocks disrupted the pattern, consistent with our observation that the *en* expression pattern is undisturbed by later heat shocks.

Autoregulation becomes dispensable

In addition to the two stages of autoregulation, we have identified one further mode of *en* regulation. Between 1 and 2 h after the transition to *wg* independence, *en* expression also becomes independent of *en* function. The evidence for later, *en*-independent regulation comes from late heat shocks of *en* mutants carrying *hs-en*. The early heat-shock protocol does not induce stable expression of the endogenous *en* locus in *en^{CXI}* homozygous embryos (see above), presumably because the mutant *en* protein cannot autoregulate. But later heat shocks



FIG. 4 Decay of *wg* RNA after heat shock of *hs-en* embryos. Embryos (125 $\times$) are hybridized with a digoxigenin-labelled³⁰ *wg* probe. *a*, Wild-type embryo, ~5 h AEL, showing the normal pattern of *wg* RNA expression³¹. *b*, An *hs-en* embryo, heat shocked at ~3 h, then allowed to recover at 25 °C for 1 h. Although the patches of *wg* expression in the head are of normal intensity (arrowhead, compare with *a*), the striped expression in the ectoderm is dramatically reduced (for comparison, arrow points to the stripe that corresponds to the arrow in *a*). Signal persists mostly in the ventral ectoderm. *c*, An *hs-en* embryo, heat shocked at ~3 h and allowed to recover for 2 h at 25 °C. There is normal *wg* staining in the head (arrowhead), but all striped ectodermal RNA has decayed (arrow shows the normal position of the stripe shown by arrows in *a* and *b*).

METHODS. Embryos were fixed and probed for *wg* RNA as in ref. 30. The *wg* probe was made from a 1.3-kb genomic *EcoRI*-*HindIII* fragment containing the fourth and fifth exons (provided by N. Baker). In a collection of embryos from parents that were heterozygous for the *hs-en* insert, only 3/4 showed premature loss of *wg* RNA after heat shock, consistent with the loss resulting from *hs-en*, due to either heat-shock-produced *en* or stable ectopic *en*.

(between about 4 and 7 h) result in reactivation of the *en*^{CXI} locus (Fig. 7a). The mutant *en* protein is expressed in a pattern of partial stripes in cells at the position expected for *en* expression. The position of the original *en* cells can be identified by a stable *lac-Z* protein expressed under the control of *en* regulatory sequences¹⁷. Though the initial expression of the endogenous *en*^{CXI} gene decays (Fig. 2d), the more stable β -galactosidase persists to the time when heat shock has reinduced *en*^{CXI} expression. The newly induced *en*^{CXI} expression is in a subset of the cells marked with *lac-Z* (data not shown).

The late *en*^{CXI} expression initially requires *en* function as it is reactivated by the pulse of *en* produced by heat shock. As reactivation is in the appropriate cells, it seems that the factors that restrict autoregulation to these cells act independently of *en* function. Heat-shock-produced *en* quickly decays, but *en*^{CXI} expression is maintained throughout embryogenesis in the absence of *en* function. This identifies a late-acting, *en*-independent maintenance programme for *en* expression.

Autoregulation not only becomes dispensible for *en* maintenance, but seems to stop functioning near the time that these new regulators come into play. As described above, heat shock of

en mutant embryos between 4 and 7 h can induce expression that lasts well beyond 7 h. But heat shocks done still later, after 7 h, do not initiate new *en* expression. The inability of the *hs-en* protein to activate the endogenous *en*^{CXI} allele shows that *en* autoregulation is not sufficient to activate *en* after 7 h.

A second indication that *en* autoregulation stops near 7 h comes from looking at the period of expression of a construct in which *en* regulatory sequences drive *lac-Z* expression in the *en* pattern¹⁷ (Fig. 7c). The construct appears to include an *en* autoregulatory element. When wild-type embryos carrying both the *en-lac* fusion gene and *hs-en* are given an early heat shock, expression of *lac-Z* is induced in novel cells in parallel with induction of the endogenous *en* gene (Fig. 7b). Hence, this construct responds to *en* autoregulation. If *en* autoregulation operates continuously, expression of this *en*-responsive construct should always parallel expression of *en*. But in wild-type embryos, *lac-Z* expression stops at about 7 h¹⁷ even though *en* expression persists. Taken together, the data indicate that *en* autoregulation does not function beyond 7 h and that *en* expression is maintained past this time by new regulators.

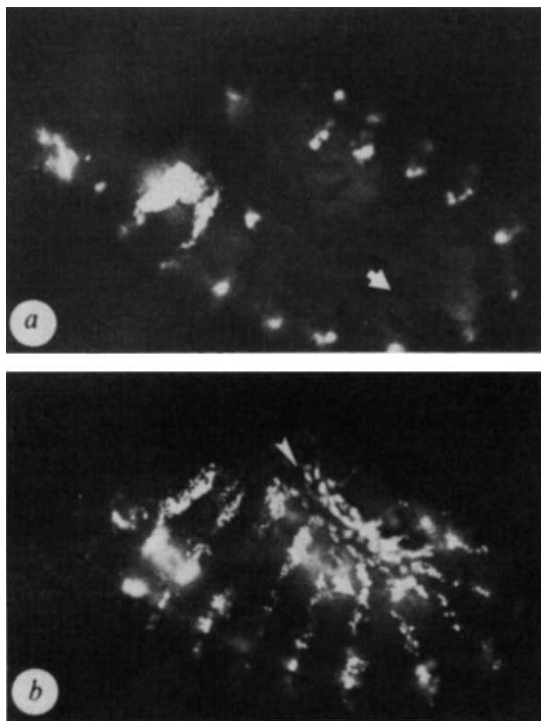


FIG. 5 Late autoregulation is *wg* independent. Embryos (140 $\times$) are stained with an anti-*en* antibody. *a*, A *wg*^{CX4}/*wg*^{CX4} embryo⁸, ~8 h AEL. There is no striped *en* expression in the ectoderm posterior to the third *en* stripe. For example, the arrow points to the normal position of *en* stripe 7. The repeated staining in the ventral region is in the CNS¹¹. *b*, A *wg*^{CX4}/*wg*^{CX4}; *hs-en* embryo, ~8 h AEL. Embryo was heat shocked at ~5 h then fixed after 3 h recovery at 25 °C. There are partial *en* stripes in positions that have no signal in a *wg* mutant without heat shock. For example, the arrow points to the partially rescued stripe 7. The staining phenotype varied. The embryo shown is representative of about half of the *wg*^{CX4}/*wg*^{CX4}; *hs-en* embryos heat shocked at this stage. The others had relatively fewer ectodermal cells staining. As with wild-type *hs-en*, there is staining in cells of the amnioserosa (arrowhead).

METHODS. Embryos were derived from parents that were heterozygous for *wg*^{CX4} on chromosome II and *hs-en* on chromosome III. Thus, 3 out of 16 of the embryos are homozygous for the *wg* mutation and carry at least one copy of *hs-en*. Consistent with this, 3 out of 16 of the embryos show the phenotype we interpret as *wg*^{CX4}/*wg*^{CX4}; *hs-en*, that is dramatically fewer than the wild-type number of *en* cells staining, due to lack of *wg*, and *en* staining in the amnioserosa, due to activity of the *hs-en* insert.

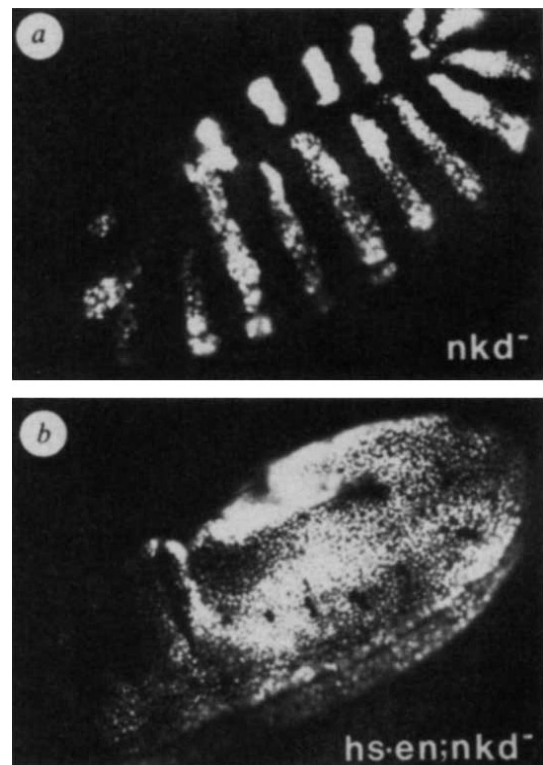


FIG. 6 Autoregulation is repressed by *nkd*. Embryos (150 $\times$) are stained with an anti-*en* antibody. *a*, A *nkd*^{TE}/*nkd*^{TE} embryo, ~5 h AEL, showing abnormally broad *en* stripes compared with wild type¹². The broadening is due to abnormal *en* expression in cells posterior to the normal *en* stripe in *nkd* mutants (S.D., unpublished observation). *b*, A *nkd*^{TE}/*nkd*^{TE}; *hs-en* embryo heat shocked at ~3 h and fixed after recovering for 2 h at 25 °C. Nearly all ectodermal cells stain except areas of the head and telson. In addition, some isolated areas in the ventral-lateral ectoderm do not express *en*. These cells vary somewhat in number and position from embryo to embryo, and thus do not appear to be cells of one specific type.

METHODS. The *nkd*^{TE}/*nkd*^{TE}; *hs-en* embryos came from parents that were heterozygous for *nkd*^{TE} on chromosome III and homozygous for *hs-en* on chromosome II. Thus, 1/4 of the embryos were homozygous for *nkd* and carried *hs-en*, and 1/4 of the embryos showed global staining in the ectoderm after recovery from heat shock. The *nkd*^{TE} stock was obtained from the Tübingen stock collection.

Progression to determination

Regulation of *en* expression is unexpectedly complex during embryogenesis. Over a 4 h period, there are four distinguishable modes of *en* activation (Fig. 8a). These regulatory programmes act in an overlapping progression. The pair-rule regulatory programme initiates striped *en* expression at cellular blastoderm^{18,19}, and we infer that it remains active until about 4.5 h, as *en* expression is maintained until this time in *wg* mutants^{11,12}. Even before 4.5 h, *wg* provides an activating signal from the neighbouring cell. This is inferred from the earliest time that *wg*-dependent ectopic *en* induction can be observed, at about 3 h. In contrast to the redundancy between pair-rule and *wg* control, *en* autoregulation is dependent on the *wg* signal during their period of overlap.

The initial dependence of autoregulation on the extracellular *wg* signal indicates that the activation of *en* expression does not immediately constitute a determined state. Instead, the period of *wg* dependence seems to represent a phase in development during which the choice of cell fate is responsive to neighbouring cells. Such a stage might provide a proofreading step to correct mistakes in the position of *en* activation. This would not be possible if *en* autoregulation were sufficient for cell-intrinsic stabilization of expression as soon as *en* is activated.

Temperature shift experiments suggest that the end of *wg* involvement in *en* expression is about 5 h. Recently, others have arrived at a similar conclusion (A. Besjovec and A. Martinez Arias, manuscript submitted). Despite the initial dependence on the *wg* signal, the *en* autoregulatory loop continues to function beyond the *wg*-dependent period, as demonstrated by the induction of *en* expression in *wg* mutants after late heat shocks. There are two likely explanations for the transition to *wg* independence (Fig. 8b). First, if *wg* acts positively, *wg*-independ-

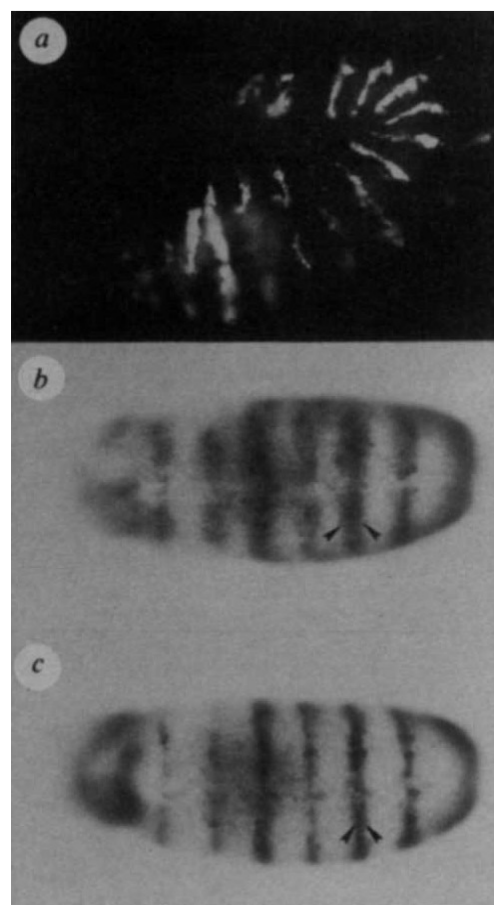
ent *en* autoregulation could arise because another positively acting factor takes over at 5 h. Alternatively, *wg* might be required to counteract an early acting repressor of *en* autoregulation. If the repressor normally decays at 5 h, the requirement for *wg* would be lifted. Whether *wg* acts positively or counteracts negative regulators of *en* autoregulation, the factors that influence *en* autoregulation do change near the time that *en* becomes *wg*-independent. This is indicated by the observation that late autoregulation is active in only a subset of the cells that are capable of early autoregulation.

Evidence for positive autoregulation has been obtained for several genes involved in the determination of cell fate, such as the *Drosophila* sex-determination gene, *Sex-lethal*²⁰, the homeotic gene, *Deformed*²¹, and a vertebrate myoblast determination gene, *myoD*²². Here, we have shown that the selector gene *en*, responsible for the determination of posterior compartments^{3,4}, undergoes positive autoregulation. Surprisingly, *en* autoregulation does not persist throughout the period that *en* is expressed. We define the end of autoregulation as the time after which heat shock cannot induce the endogenous allele in *en* mutants, and the time that an *en*-responsive reporter gene loses expression in a wild-type background. As autoregulation is transient, it does not supply a mechanism for stable determination of the *en* cell fate.

We have detected a yet-later-acting tier of *en* maintenance that is independent of autoregulation (Fig. 8a). After the *wg*-dependent period, the transient production of functional *en* through heat shock promotes the stable expression of the *en*^{CXI} mutant protein in homozygous embryos. Thus, after an autoregulatory phase, *en* is controlled by new regulators that maintain expression independent of *en* function. This program may provide maintenance of expression and not activation as *en*^{CXI} expression is not reactivated at late stages without heat shock.

FIG. 7 A fourth mode of *en* control follows autoregulation. *a*, An *hs-en;en*^{CXI}/*en*^{CXI} embryo (110×), heat shocked at ~5 h, aged 3 h, then fixed and stained with an anti-*en* antibody. The cytoplasmic *en*^{CXI} protein is expressed in partial stripes in each segment. *en*^{CXI} mutants normally show no expression by this stage. The number of cells that are rescued by heat shock varies, depending in part on the age at heat shock. This embryo is representative for a heat shock done at 5 h. Most rescued cells lie in the lateral and dorsal ectoderm and are a subset of the cells that initially express *en*^{CXI} in early embryos (see text). *b*, An *en-lacE;hs-en* embryo (75×, ventral view, anterior left) heat shocked at ~3 h, aged 2 h, then fixed and hybridized with a digoxigenin-labelled³⁰ *lac-Z* probe. Stripes of *lac-Z* RNA are abnormally broad compared with non-heat shocked control embryos (compare distance between arrowheads flanking the stripes in *b* and *c*). *c*, An *en-lac-E* embryo (75×, ventral view, anterior left), fixed at ~5 h and hybridized with a digoxigenin-labelled³⁰ *lac-Z* probe. The *en-lac-E* contains 2.4 kb of *en* promoter/upstream sequences and the *en* first intron driving *lac-Z* expression¹⁷. Stripes of *lac-Z* RNA correspond to the position of *en* expression, although the level of *lac-Z* expression from this construct varies among the stripes.

METHODS. *a*, Embryos were derived from parents that were heterozygous for *en*^{CXI} on chromosome II and *hs-en* on chromosome III. Thus, 3/16 of the embryos are homozygous for the *en* mutation and carry at least one copy of *hs-en*. Consistently, 3/16 of the embryos show the phenotype we interpret as *en*^{CXI}/*en*^{CXI}; *hs-en*, that is cytoplasmic stain in ectodermal stripes with fewer than the normal number of cells staining in each stripe. *b*, *c*, Embryos were fixed and probed for *lac-Z* RNA as in ref. 30. The *en-lac-E* stock was obtained from J. Kassis.



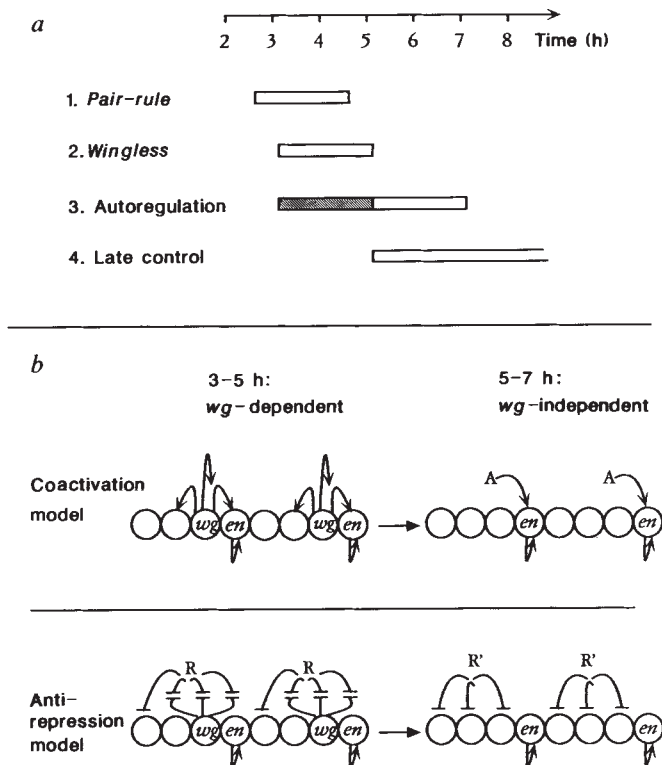


FIG. 8 Integrating four modes of *en* regulation during embryogenesis. *a*, Approximate periods of action of the four modes of *en* regulation and their temporal overlap. The time line indicates hours AEL. 1, The start of the *pair-rule* period is defined as the time of the earliest appearance of *en* expression^{7,14}. The end is defined as the time when *en* expression stops in *wg* mutants¹¹, presumably a condition in which only *pair-rule* function activates *en*. 2, The start of *wg* influence is defined as the earliest time that heat shock stably induces *wg*-dependent ectopic *en* expression. The end of the *wg* period is defined as the latest time at which raising the temperature of *wg*^{ts} embryos has an effect on *en* expression. 3, The start of autoregulatory influence is taken as the earliest time that heat shock stably induces ectopic *en*. The end of the period during which *en* autoregulation operates is inferred from two observations: first, that *en*-responsive sequences cannot drive *lac-Z* expression past this time, and second, that heat shock does not activate expression in *en* mutants past this time. Stippling indicates the time during which autoregulation requires *wg* input. 4, The fourth mode of regulation (late control) begins when heat shock first results in stable expression in *en* mutants. It is not known whether this regulatory phase is responsible for maintaining *en* indefinitely or whether still later transitions occur. *b*, Two models to account for the onset of *wg*-independent *en* autoregulation. Cells spanning two segment primordia are schematized as eight open circles. Labelled cells represent those that normally express *en* or *wg*. Coactivation model: cells directly exposed to the *wg* coactivation signal are competent to autoregulate during the *wg*-dependent period. Autoregulation is allowed in whichever of these cells *en* is activated, either by *pair-rule* gene activity^{18,19} or by heat shock. Expression of *en* becomes *wg*-independent when another coactivator (A) takes over. This new coactivation is confined to posterior compartment cells, and *en* expression is thereby restricted to the posterior compartment after late heat shock. Antirepression model: *wg* prevents a general repressor of *en* (R) from acting on *wg* cells and their neighbours during the *wg*-dependent period. Autoregulation is allowed in whichever of these cells *en* is activated, either by *pair-rule* gene activity^{18,19} or by heat shock. Later, the *wg* requirement is relieved because late repression (R') is confined to the anterior compartment. Autoregulation of *en* can now act in the normal *en*-expressing cells in the absence of the *wg* signal. Late heat shock cannot overcome the late repression in the anterior compartment and so does not induce ectopic autoregulation. It should be noted that, although we suggest that ectopically induced *en* expression occurs in the *wg*-expressing cells, we have not yet experimentally identified which cells induce *en* after heat shock. Additionally, *wg* is still expressed at late stages, but has not been indicated in the schematic because it is dispensable for *en* expression.

Although it has not yet been tested directly, it may be this level of *en* regulation that provides the stable, cell intrinsic control that characterizes a determined cell. Candidates for later acting *en* regulators, both positive and negative, have been identified among the *trithorax* and *Polycomb* gene classes. Mutations in these genes have been found to affect maintenance of expression patterns of homeotic selector genes²³⁻²⁵, and members of the *Polycomb* class have been shown to affect *en* expression as well (D. Moazed and P.O'F., unpublished data)²⁶⁻²⁸.

The classically defined stages of reversible and irreversible

cell fate commitment lead us to expect changes in the mode of *en* regulation during development. But the elaborate progression that we have uncovered is difficult to rationalize in all its detail. Although the transition to *wg* independence suggests a move away from cell-extrinsic control of identity, the subsequent transition, from autoregulatory activity to a still later stage of control, has no ready explanation. Identifying the later-acting regulators and the time at which the *en* cell fate becomes irreversibly determined may help explain these transitions. □

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A dynamical instability of bars in disk galaxies

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STRONG, rapidly rotating, persistent bars readily form in numerical simulations of initially axisymmetric disk galaxies¹⁻⁴. The global dynamical instability responsible for this behaviour is a great embarrassment to the subject of galactic dynamics, as most galaxies in the sky do not possess a strong bar⁵; the suggestion³ that the dark matter content of galaxies might resolve this discrepancy is unattractive⁶. Here we present the results of three-dimensional simulations which show that the bar is dynamically unstable to buckling out of the galactic plane (the majority of previous disk galaxy simulations have been strictly two-dimensional). Stars acquire large motions normal to the plane, giving the bar a peanut shape through a mechanism that we suggest to be the fire-hose instability⁷⁻⁹. Bars may be weakened or even destroyed by this instability; thus the fraction of disk galaxies containing strong bars today could be lower than the fraction in which they have formed in the past. The instability may also account for the peanut morphology of many galaxies, and because it leads to a less flattened stellar system with increased central density, it may play a part in the formation of galactic bulges.

We report two fully self-consistent three-dimensional particle-mesh simulations of a disk-halo galaxy. The distribution function for the spheroidal component is generated iteratively⁹, to ensure that both halo and disk are initially in equilibrium. We use a Kuz'min/Toomre disk¹⁰ containing 60% of the total mass, sufficient to ensure bar formation. The remainder of the mass is in the spheroid, which approximates a truncated Plummer model with length-scale equal to that of the disk. The disk is largely rotationally supported, but particles have an initial velocity dispersion in the plane 1.3 times that required for local axisymmetric stability¹¹.

The two models discussed here differ only in their initial disk thickness; the disk aspect ratio is 50:1 in the first model and 10:1 in the second. These are reasonable values for respectively young and old stellar populations in galactic discs. We use a

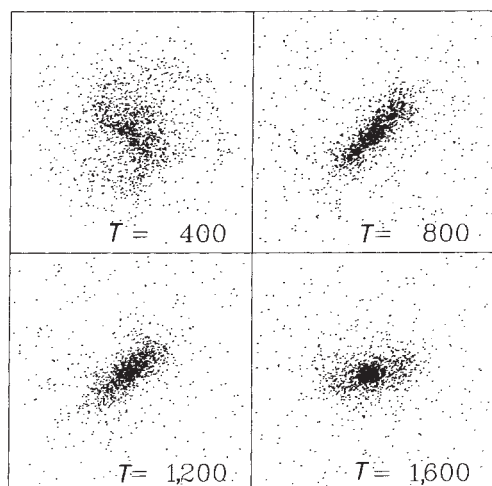


FIG. 1 Pole-on views of the evolution of the thin disk. Boxes indicate the edge of the cartesian grid, which is 8.0 Kuz'min/Toomre disk scale lengths on each side, and the rotation period at the end of the bar is ~ 200 of our time units. The bar takes about four orbital periods to form, but after the buckling instability (see text) has run its course, the bar is weaker and more centrally concentrated (time $T=1,600$).

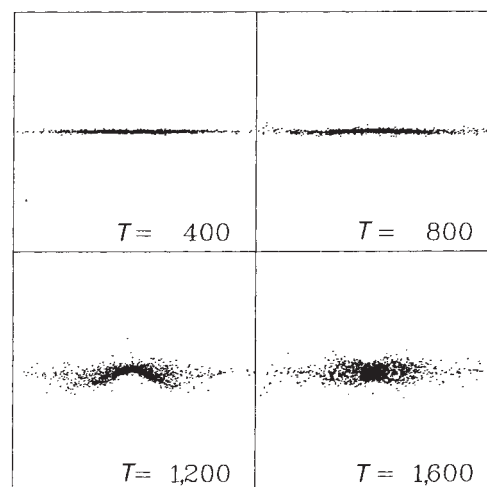


FIG. 2 Side-on views of the evolution of the thin disk seen from the minor axis of the bar. The bend, which is already visible at $T=800$, becomes spectacular by $T=1,200$. The bar later settles back to the plane, but with pronounced thickening into a persisting peanut shape ($T=1,600$).

total of 1.5×10^5 particles and calculate potentials on a cubic cartesian grid with 62 cells in each direction¹²; the code has been extensively tested¹³. As an additional check of the code, and to verify our result at higher spatial resolution, we used a tree code¹⁴ kindly supplied by L. Hernquist. As this code is much less efficient, we use just 1.5×10^4 particles for the check. The smaller softening length led to quantitative changes but the qualitative behaviour, including the buckling instability described below, was identical. Although this successful check considerably enhances our confidence in the main result, we recognize that still higher resolution would be desirable.

Figure 1 shows four widely spaced face-on views of the disk particles in the thin disk model, illustrating the formation and the subsequent almost complete disruption of the bar. Figure 2 shows side views from a point on the minor axis of the bar. Soon after the bar develops, it begins to bend out of the disk plane, reaching a considerable maximum distortion (Fig. 3; note the saddle shape). The exponential growth rate of this buckling mode is about as rapid as the planar bar instability itself.

After the stage of maximum distortion, the centre line of the bar, as measured by the mean z -displacement of particles, briefly develops structure on shorter scales, and then settles back to the galactic plane. The energy in the buckling mode cascades to shorter spatial wavelengths, before raising the velocity dispersion in the perpendicular direction, and thus increasing the thickness. This effect is most pronounced towards the ends of the bar where displacements from the plane are greatest. During this phase, the bar pattern abruptly weakens while the central density of the model rises. The angular rotation rate of the bar does not, however, change much.

We present results from a thick disk simulation in Fig. 4. The bar instability develops later in the run and is again followed by a (less violent) buckling instability. The mean z -displacement of the particles evolves in a similar manner to that in the thin disk model, and again the bar develops a 'butterfly shape' when viewed from the side. Because the bending instability is less violent, it causes less damage to the bar and disk.

We interpret the bending behaviour as an example of the well known 'fire-hose' instability. Toomre and others^{7,8,15} concluded that this is unlikely to occur in a rotationally supported stellar disk unless the velocity ellipsoid is very flattened. Bar formation, however, makes the orbits of stars in the bar much more eccentric, and aligns their principal axes to lie close to the bar major axis, leaving the vertical motions largely unaffected (Fig. 2).

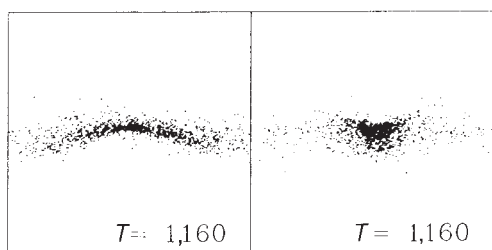


FIG. 3 Side and end views of the thin disk bar at $T=1,160$, detailing the saddle shape formed at the moment of greatest bending. The scale is twice that of Figs 1 and 2, and the boxes do not correspond to the grid boundary. The abscissa in the left picture is parallel to the major axis of the bar, whereas that in the right picture is parallel to the minor axis.

The bar is therefore more vulnerable to buckling instabilities than was the original axisymmetric disk.

The mutual gravitation of stars in the bar couples their motion and preserves the coherence of the system to slow distortions. We can liken the bar to a hollow rotating tube which confines the stars to its interior. The weakest point is at the centre where the stars move most rapidly and the centrifugal effect is greatest; notice in Fig. 3 that the bend is sharpest at the centre. The ratio of velocity dispersion normal to the bar plane to r.m.s. velocity along the bar (mass-weighted across the minor axis) is 0.13 for the thin disk model and 0.39 for the thick disk case. Although local theory⁸ suggests that bending modes are stabilized when the ratio exceeds 0.29, it is hardly surprising that it fails in this very nonuniform situation. Local values of the ratio for the thick disk vary from 0.24 on the edges of the bar (where the bending is most pronounced) to 0.57 at the centre.

A similar instability has been observed by Merritt and Hernquist¹⁶ in nonrotating highly prolate models of elliptical galaxies. As with our thick disk model, they observe buckling in systems with much less extreme velocity anisotropies than those required for the fire-hose instability in a slab⁷ or a disk⁸.

Combes *et al.*¹⁷ observe bar thickening in simulations very similar to ours, and attribute it to orbit trapping. The orbit family they identify is a promising candidate to account for the final peanut shape of their bar, but we suggest that the fire-hose

instability promoted its development in their models too. Our conjecture is supported by the report¹⁸ that no thickening occurs in their models if reflection symmetry is imposed about the galactic plane.

This instability may be relevant to three current problems of galactic structure. First, it offers a natural explanation for the box or peanut morphology observed in the light distributions of many nearly edge-on systems¹⁹. Second, it increases the thickness and central surface density of the disk; the combined bar-forming and disruption instabilities may therefore offer a new way of making galactic bulges without mergers or star formation during initial collapse of the galaxy. (The very high central concentrations of bulges cannot be realized in a numerical simulation of this resolution. A dissipative gas component, which we have yet to include, could further increase the central density.) Third, by providing a means of weakening bars, it implies that galaxies lacking bars today might nevertheless have experienced bar instabilities in the past. Bars in thin, newly-formed disks are particularly fragile. The incidence of the buckling instability in galaxies, together with detailed comparison of the final configurations with observations, will be discussed elsewhere. □

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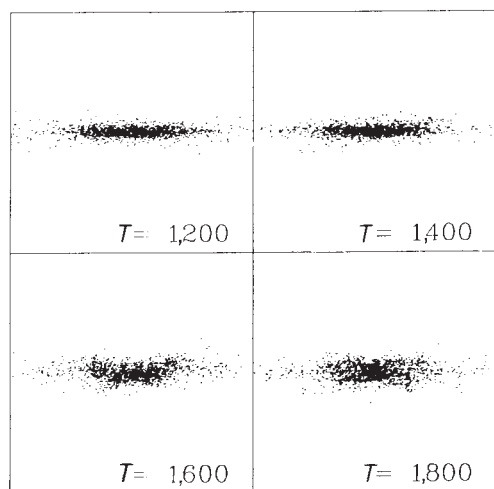


FIG. 4 Side-on views of the evolution of the thick disk bar in its rotating frame. Axes and dimensions are as in Fig. 2. The bend is already clearly noticeable by $T=1,400$ and the maximum bend, less marked than in Fig. 2, is reached at $T=1,560$ (not shown). Again the bar settles back to the plane with a thick peanut shape ($T=1,800$) but with less marked central concentration.

Comparison of a calculated spectrum of $C_{60}H_{60}$ with the unidentified astronomical infrared emission features

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INFRARED emission features consisting of narrow lines and broad plateaux, ranging from $3\mu\text{m}$ in wavelength to over $12\mu\text{m}$, are seen in a wide variety of astronomical objects in which interstellar or circumstellar gas is illuminated by ultraviolet radiation from a star. Several candidates have been proposed as the source of this emission, but none is widely accepted. Here I calculate the vibrational spectrum of the fullerane $C_{60}H_{60}$, the saturated hydride of the soccerball-shaped molecule C_{60} , using a force-field model. Six of the infrared active frequencies match unidentified emission lines to within 4%, and a seventh differs from an observed line by 8%. The calculation suggests why the astronomical feature at $7.7\mu\text{m}$ is the strongest, and the observed variation of the $3.4\mu\text{m}$

and 3.28 μm features with astrophysical environment is consistent with the idea that the former is attributable to stretching of the C-H bond in heavily hydrogenated fullerenes, whereas the latter is due to the same transition in lightly hydrogenated fullerenes.

The features have been known since 1973^{1,2}, and the strong lines at 3.28, 3.40, 6.2, 7.7, 8.6 and 11.3 μm have been well studied^{3,4}. Other features have since been discovered, including a weak feature⁵ at 12.7 μm , which was hidden by a line of Ne II, and one at 6.9 μm , which is only strong in objects of low excitation^{6,7}. The carrier is almost certainly carbon-rich⁸, and many possible identifications have been put forward, but none is widely accepted as being entirely satisfactory. In reviewing the existing proposals, Sellgren⁹ has pointed out a severe difficulty with all of them: the identification of the 7.7- μm feature is the most problematical, yet it is observed to be the strongest by far.

As the fullerenes (hollow, spherical carbon shells) and the fullerenes (the hydrides of the fullerenes) are newly discovered families of carbon-rich molecules, it is natural to enquire whether they might be the carrier. It has already been suggested^{10,11} that C_{60} and C_{60}^+ are important in astrophysics, but the infrared spectrum of C_{60} is known¹² and does not match the unidentified infrared features. The fullerenes and their ions are also of interest, but their infrared spectra are not known. To look at the general features of the spectra of these molecules, I decided to calculate the vibrational properties of one of them (A.W., in preparation). I selected $\text{C}_{60}\text{H}_{60}$ because all the chemical bonds are of one well-understood type, namely covalent. A force-field model¹³ was adopted for the calculation, with the carbon atoms located at the vertices of a regular, truncated icosahedron as in the usual model¹⁰ for C_{60} , and with the hydrogen atoms positioned radially outwards from them: the structure has the symmetry of I_h , the icosahedral group with inversion¹⁴. The model is fully defined when the bond lengths are specified and when the stretching and bending stiffness constants of the bonds are given. Standard values of the bond lengths and elastic constants were taken¹⁵ (Table 1), leaving no parameter free or adjustable, and the dynamical problem was solved by a standard method¹⁶. Of the 354 vibrational modes, only the 27 belonging to the irreducible representation F_{1u} are infrared active; this representation is triply degenerate, so there are nine active frequencies, which are given in Table 2. Table 2 also contains a column of numbers proportional to the matrix element of the transition dipole moment, on the assumption that the carbon and hydrogen

TABLE 1 The constants of the force-field model

C-C length	1.543 Å
C-H length	1.093 Å
C-C stretch	4.307 mdyne Å ⁻¹
C-H stretch	4.623 mdyne Å ⁻¹
C-C-C bend	1.084 mdyne Å rad ⁻²
C-C-H bend	0.667 mdyne Å rad ⁻²

atoms have equal and opposite electric charges, and the wavelengths of nearby unidentified infrared emission lines, as catalogued in a recent review⁹. There are no such lines known near $1F_{1u}$ and $2F_{1u}$, but all the others have possible matches and the r.m.s. error between the measured and calculated wavelengths is 3.7%. In Fig. 1, two astronomical spectra are shown with the calculated wavelengths marked for comparison, and it can be seen that the agreement between the observed⁹ and calculated wavelengths is not attributable to there being a large number of observed lines to choose from; there are only two strong lines not accounted for in this way, at 3.28 and 12.7 μm . The poorest match is between $3F_{1u}$ and the line at 11.3 μm , which differ in wavelength by 8% or 2.2 standard deviations, but it is not clear whether this difference is large enough for it to indicate a misidentification. It would not be expected that such a simple model would reproduce the wavelengths exactly in any case, so to estimate the likely accuracy an identical calculation was performed on dodecahedrane ($\text{C}_{20}\text{H}_{20}$), a molecule of similar structure, which has been synthesised in the laboratory¹⁷. With the same choice of elastic constants and the same lack of free parameters the model gave 3.43, 7.43 and 13.9 μm for the wavelengths of the active transitions. The measured values¹⁷ are 3.40, 7.70 and 13.7 μm and differ from those calculated by 2.3%, which is similar to the agreement between $\text{C}_{60}\text{H}_{60}$ and the astronomical lines. A force-field calculation cannot be used to prove that $\text{C}_{60}\text{H}_{60}$ is the carrier of the unidentified astronomical lines, but if they are carried by $\text{C}_{60}\text{H}_{60}$ then the calculation of the wavelengths evidently matches the observations about as well as might be expected.

According to present ideas, an interstellar cloud is likely to contain a wide variety of fullerenes and not just $\text{C}_{60}\text{H}_{60}$, so it is of interest to find out whether anything can be inferred about

FIG. 1 The active wavelengths of $\text{C}_{60}\text{H}_{60}$ from the calculation compared with spectra (flux, F_λ , against wavelength) of the unidentified infrared emission in two astronomical sources. Along the horizontal line labelled ' $\text{C}_{60}\text{H}_{60}$ ' are marked the wavelength and symmetry symbol of each of the active modes of the molecule. Above it is the classic spectrum² of NGC7027 with the wavelengths of the unidentified features marked; it shows all the features mentioned in the text although the line at 6.9 μm is weak. This line is much stronger in the spectrum of IRAS22272, which is shown below for comparison⁷. Spectral lines securely identified with transitions of atoms and atomic ions have been deleted from these spectra for clarity.

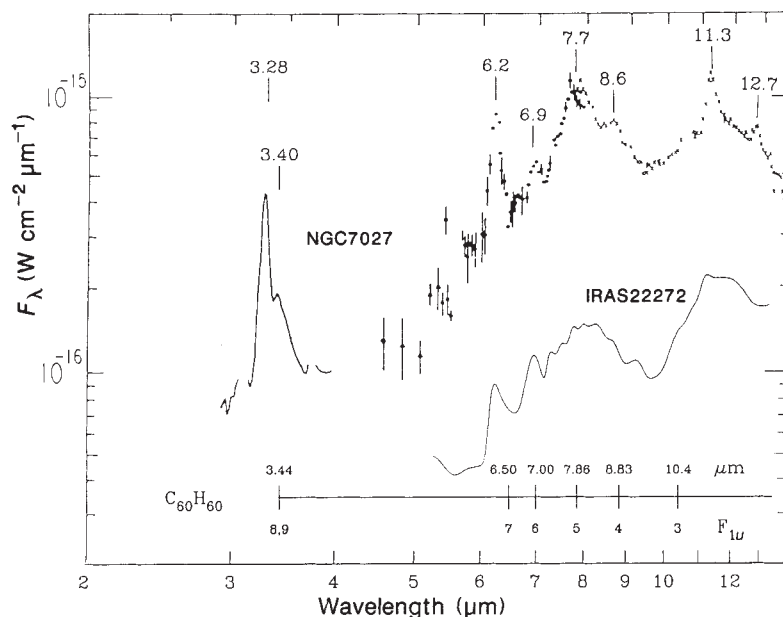


TABLE 2 Tentative assignment of calculated wavelengths of $C_{60}H_{60}$ to unidentified infrared features

Mode	Calculated dipole	$\lambda(\mu\text{m})$	Interstellar $\lambda(\mu\text{m})$
1F _{1u}	1.6	23.0	—
2F _{1u}	0.5	20.5	—
3F _{1u}	0.4	10.4	11.3
4F _{1u}	0.8	8.83	8.6
5F _{1u}	5.5	7.86	7.7
6F _{1u}	1.5	7.00	6.9
7F _{1u}	0.3	6.50	6.2
8F _{1u}	2.7	3.44	3.40
9F _{1u}	0.01	3.43	3.40

their spectra from the calculation. Many of the modes of $C_{60}H_{60}$ are likely to be peculiar to that species and not to be related to the modes of other fullerenes, but the group modes are different. These modes are dominated by the deformation of just one type of elastic structure and are likely to be present in all the fullerenes, because all the fullerenes possess that structure. There are three group modes: 5F_{1u}, dominated by the bending of C–C–H angles, and 8F_{1u} and 9F_{1u}, dominated by the stretching of C–H bonds. The interstellar clouds are therefore expected to radiate well at 7.7 and 3.4 μm . The mode 5F_{1u} is special in another way: it has the greatest transition dipole moment and should radiate most strongly. It is likely that the corresponding modes in the other fullerenes will also radiate strongly, so it seems that Sellgren's criticism⁹ may not apply: the feature near 7.7 μm would naturally be the strongest if the fullerenes are the carrier.

There are 60 fullerenes of the family $C_{60}H_m$ ($m = 1, \dots, 60$), or $\sim 10^{16}$ when account is taken of isomerism (different arrangements of the hydrogen atoms). Most of the radiation from a cloud containing all of them would therefore be expected to consist of a blend of many lines which would resemble a continuum over a wide range of wavelengths; such a quasi-continuum might explain the plateaux in the unidentified infrared features. The blending may also determine the line profiles and their widths, because near each line of $C_{60}H_{60}$, lines of $C_{60}H_{59}$ are expected, and a little further away lines of $C_{60}H_{58}$ and so on, giving a blended profile whose parameters would be determined by the vibrational properties of the neighbouring species and not by those of $C_{60}H_{60}$ itself. There are many ways in which the populations of two different clouds of fullerenes could differ: in the average degree of hydrogenation, for example, or in the excitation. It is therefore not surprising that the spectrum of the unidentified infrared features varies from source to source (Fig. 1).

The calculation indicates that $C_{60}H_{60}$ is not the carrier of the line at 3.28 μm , and as this is the best studied of all the features, it is of particular interest to enquire what is. The carrier may not be a fullerene at all, but if it is, then it must be very different from $C_{60}H_{60}$, because it is unlikely that C–H stretch in any predominantly single-bonded (aliphatic) hydrocarbon could radiate at such a short wavelength. A hint may be found in the difference in the spatial behaviour of the 3.28 and 3.40 μm lines in, for example, the Orion Bar and HD44179, where the flux ratio (flux at 3.40 μm)/(flux at 3.28 μm) increases with distance from the exciting stars¹⁸. On the simple assumption that the degree of hydrogenation of the fullerenes increases with distance as the effect of photodissociation decreases, the variation of the flux ratio is much as would be expected if the lines at 3.40 and 3.28 μm are carried by C–H stretch in heavily and lightly hydrogenated fullerenes respectively. In a lightly hydrogenated fullerene such as $C_{60}H$ or $C_{60}H^+$, the bonding is aromatic throughout most of the molecule, and we cannot be certain that the vibrational frequency of the C–H group will be the

same as that in a completely aliphatic molecule. If it is allowed that the wavelength might be different and that the astronomical measurements may be taken as a guide, there is a case for supposing that the wavelength of C–H stretch in a fullerene with only a few hydrogen atoms is 3.28 μm . Granted this supposition, the flux ratio of the lines at 3.40 and 3.28 μm becomes an observational index of the degree of hydrogenation of the fullerenes in the astronomical sources. □

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Highly oriented thin films of poly(tetrafluoroethylene) as a substrate for oriented growth of materials

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THE formation of highly oriented structures such as single crystals, single-domain liquid crystals and systems comprising uniaxially oriented crystallites is important in many applications of thin films and interfaces, ranging from materials reinforcement to molecular electronics. Of the methods that exist for forming such oriented structures, however, few have sufficient generality to make them applicable to materials of differing chemical composition or physical properties. Here we present a simple and surprisingly versatile method¹ for orienting a wide variety of crystalline and liquid-crystalline materials, including polymers, monomers and small organic and inorganic molecules. In our technique, a thin, single-crystal-like film of poly(tetrafluoroethylene) (PTFE) is deposited mechanically on a smooth substrate such as glass. Materials grown on this coated surface from solution, melt or vapour phases show a remarkable degree of alignment.

The commercial significance of oriented systems derives from the fact that the properties of the oriented materials may exceed those of the isotropic species by orders of magnitude. For example, increases of more than a factor of 100 have been reported for the stiffness and strength of highly oriented crystalline polymers^{2–4} and for the electrical conductivity of doped and aligned conjugated macromolecules^{5–7} compared with un-oriented materials. Amongst many other examples of improved

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performance of oriented materials are enhanced thermal conductivity⁸, piezoelectric properties⁹ and optical transparency.

A wide variety of techniques has been developed to promote oriented growth. For example, metals, polymers and small molecules not prone to form large single crystals have been processed into oriented structures through epitaxial crystallization on single-crystal substrates^{10,11} or on moderately oriented polymers^{12–14}. In isolated cases, graphoepitaxy¹⁵, which is oriented growth on substrates of supramolecular, microscopic gratings (of typical spacing $\sim 0.3 \mu\text{m}$), has been reported to be successful. Small-molecule liquid crystalline compounds, which are important in display technology, are oriented into domains by deposition on 'rubbed' surfaces of certain polymers^{16,17} and/or in magnetic or electric fields. Oriented structures of macromolecules, such as films and fibres, are generally made by mechanical deformation in the solid, gel or liquid crystalline state^{2–4}, or by epitaxial crystallization (see, for example, reviews in refs 18 and 19). Virtually all of the above techniques either produce only a limited degree of order, or are restricted to a limited range of materials. The method that we present here, however, is highly versatile, enabling oriented growth of a wide variety of materials.

The deposition of the thin, orientation-inducing layers of PTFE is relatively straightforward, and derives from detailed observations made by workers in the field of polymer wear^{20,21}. The method is schematically depicted in Fig. 1a. Electron-diffraction studies of the PTFE layers (Fig. 1b) showed, in accordance with previous observations²⁰, that the chain axis of the PTFE macromolecules was oriented parallel to the glass surface (in other words in the plane of the layer) and along the sliding direction. The observed alignment and crystalline perfection of deposited layers were very high and compared favourably with those found in extended chain whiskers of PTFE directly

formed during synthesis²² or in high-performance, gel-processed ultra-high molecular weight polyethylene²³. A transmission electron micrograph of a platinum/carbon replica of the PTFE layer is presented in Fig. 1c. This micrograph shows that the surface was not molecularly smooth, but consisted of many steps of various heights, which extended along the path of deposition.

These PTFE-coated glass slides subsequently were used as substrates for the deposition of a variety of materials. The species of interest were grown on the substrates from the melt, solution or gas phase, at temperatures below the melting point of PTFE ($\sim 340^\circ\text{C}$).

A first example is given in Fig. 2a, which shows an optical micrograph of poly(ϵ -caprolactone), PCL, crystallized from the melt onto the oriented PTFE layer, and on the adjacent bare glass surface as a reference. The left-hand side of the figure reveals the typical isotropic, spherulitic growth habit of poly(ϵ -caprolactone) crystallized on the uncoated glass. The right-hand side, by contrast, shows the striking, homogeneously oriented morphology of the polymer grown on the PTFE layer. The optical properties of the film unambiguously demonstrated that the poly(ϵ -caprolactone) macromolecules are oriented parallel to the PTFE chains.

Interestingly, precipitation on PTFE-coated substrates from solutions of various species in many cases also yielded oriented films. Illustrative examples are poly-para(phenyleneterephthalamide), which is the constituent of the high-performance aramid fibres Kevlar[®] and Twaron[®], and the electrically conducting polymer polyaniline, both of which were precipitated from concentrated (96%) sulphuric acid by exposure to moist air²⁴. An optical micrograph of the latter polymer is shown in Fig. 2b. Again, this micrograph illustrates the conspicuous structural differences between the polyaniline precipitated on the

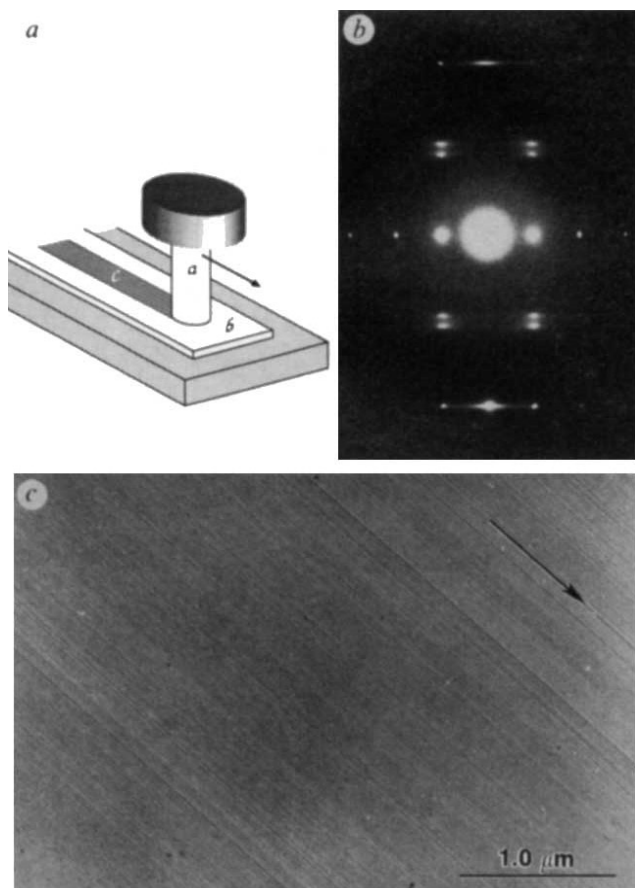


FIG. 1 a, Schematic diagram of mechanical deposition of thin layer of poly(tetrafluoroethylene) (PTFE). A bar of solid PTFE (A) is moved against a smooth counterface, for example glass (B), at controlled rate and temperature, while applying a pressure. This simple procedure leaves on the glass surface a thin layer of highly oriented PTFE (C). Typically, the temperature of the glass surface was 130°C , the sliding rate 1 mm s^{-1} and the pressure $\sim 1 \text{ kg cm}^{-2}$. Depending on the pressure, temperature and sliding rate the thickness of the deposited layer can be varied from ~ 2 – 100 nm . b, Electron diffraction pattern of the PTFE layer. The chain axes of the macromolecules are oriented along the sliding direction, which was vertical. This pattern shows the extraordinary degree of orientation and crystalline perfection of the PTFE layer. c, Transmission electron micrograph of the mechanically deposited PTFE film. The film was shadowed with platinum and carbon and backed with a carbon support layer (arrow indicates sliding direction).

FIG. 2 *a*, Optical micrograph, taken with crossed polarizers, of poly(ϵ -caprolactone) crystallized on a glass slide partially covered with a thin layer of oriented PTFE. The poly(ϵ -caprolactone) was precipitated from a CHCl_3 solution by evaporation of the solvent. Subsequently, the polymer was melted and recrystallized from the melt. The left-hand side of the photograph shows the typical isotropic spherulites observed for isotropically crystallized poly(ϵ -caprolactone). By contrast, the right-hand side of the picture displays a highly birefringent, homogeneously oriented poly(ϵ -caprolactone) structure, which resulted from growth on the PTFE layer. It should be noted that the latter, very thin (~ 10 nm) layer itself is virtually invisible in the polarizing optical microscope. Note also the presence of a marked transcrystalline zone at the boundary of the PTFE layer. *b*, Optical micrograph, taken with crossed polarizers, of polyaniline crystallized onto a thin layer of oriented PTFE. The polyaniline was precipitated from 96% sulphuric acid by absorption of moisture from the air. The left-side of the photograph shows the typical isotropic spherulites observed for polyaniline. By contrast, the right-hand side shows a highly birefringent, homogeneous polyaniline structure resulting from growth on the PTFE layer. The scale bar represents 1 mm on both micrographs. *c*, Electron diffraction pattern of a highly oriented poly(*p*-xylylene) (PPX) film in its β crystal form. The polymer film was directly synthesized from the gaseous monomer *p*-xylylene by deposition under vacuum on a glass slide coated with oriented PTFE (J.C.W., S. Graff, S. Meyer and P.S., manuscript in preparation, and ref. 26). The as-polymerized PPX film was subsequently annealed at $\sim 300^\circ\text{C}$. PPX chain axis is vertical.

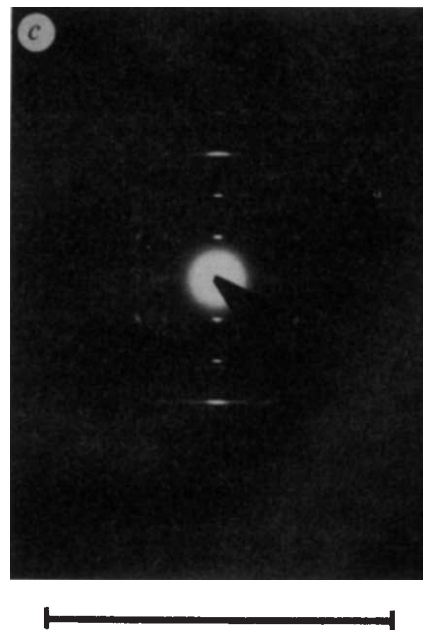
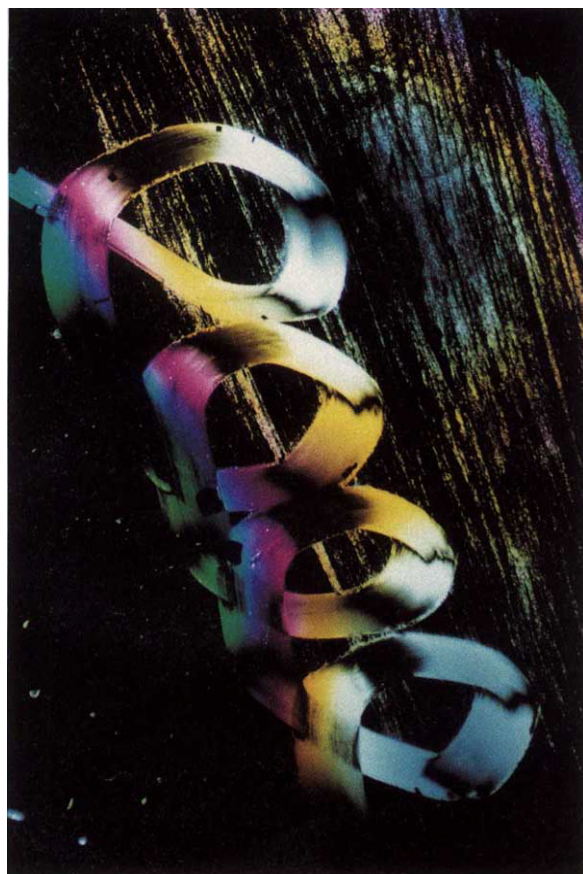
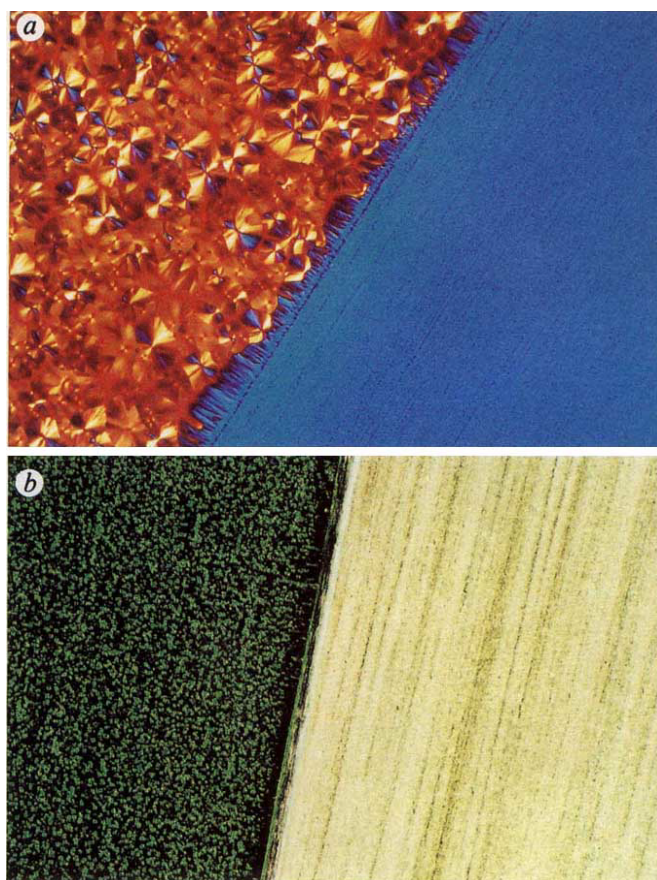


FIG. 3 Optical micrograph, taken with crossed polarizers, of the small-molecule liquid crystalline compound 4-cyano-4'-*n*-hexylbiphenyl (K 18, BDH Ltd) grown on a glass substrate that was coated with a thin layer of PTFE in a 'helical' pattern. In this case a continuous, nonlinear structure was produced by moving a pencil-shaped PTFE rod in a spiralling path across the glass substrate. The liquid crystalline compound was then applied to the decorated substrate. This optical micrograph shows that the liquid crystalline material formed domains with a continuously changing director, following the orientation of the underlying PTFE layer. Scale bar represents 1 mm.

TABLE 1 Examples of materials grown in highly oriented form on mechanically deposited thin films of PTFE

Species	Conditions*
Small organic	
adipic acid	vapour
anthraquinone	melt, 200 °C
chloranil (tetrachloro- <i>p</i> -benzoquinone)	vapour
alkanes	melt, 100 °C
perfluoroalkanes	melt, 180 °C
2-methyl 4-nitroaniline	vapour
Inorganic	
thallium chloride	vapour
Liquid crystalline	
mixtures of 4-cyano-4'- <i>n</i> -alkylbiphenyls	melt, 80 °C
4- <i>n</i> -propyl-cyclohexyl-4'-ethoxybenzene	melt, 100 °C
poly-[4-cyanophenyl 4-(6 acryloyloxy-hexyloxy) benzoate]	melt, 150 °C
Monomer	
para-xylylene, polymerized into poly(<i>p</i> -xylylene)	†
Polymer	
poly(tetrafluoroethylene) oligomers	melt, 200 °C
poly(ethylene terephthalate)	melt, 270 °C
poly(butylene terephthalate)	melt, 260 °C
polyethylene	melt, 160 °C
nylon 6	melt, 280 °C
nylon 11	melt, 200 °C
poly(1-butene)	melt, 150 °C
poly-ε(caprolactone)	melt, 100 °C
polyaniline	5 wt% solution in H ₂ SO ₄ , 25 °C
poly-para-(phenylene terephthalamide)	2 wt% solution in H ₂ SO ₄ , 25 °C

* Conditions refer to the method of deposition.

† J.C.W., S. Graff, S. Meyer and P.S., manuscript in preparation; see also ref. 26.

orienting PTFE layer and on the uncoated glass surface. These examples illustrate an important advantage of the present PTFE orienting layers, which is their excellent resistance against most known chemicals, including highly aggressive acids.

Oriented polymer structures were obtained not only by crystallization but also by direct polymerization of a monomer on the PTFE orienting layers, as is exemplified by poly-*p*-xylylene (PPX) (J.C.W., S. Graff, S. Meyer and P. S., manuscript in preparation). An electron diffraction pattern showing the outstanding uniaxial orientation of the polymerized PPX macromolecules is shown in Fig. 2c.

Figure 3 illustrates the use of the orientation-inducing PTFE layers to form ordered structures of small-molecule liquid crystalline compounds. Featureless mono-domains of certain liquid crystals were observed when deposited on uniaxially oriented PTFE layers. The micrograph of Fig. 3 shows a structure manufactured by depositing PTFE in a continuous, nonlinear pattern, and subsequently applying the liquid crystalline compound 4-cyano-4'-*n*-hexylbiphenyl. This optical micrograph shows that the latter compound formed domains with a continuously changing director (principal axis of orientation), following and, in fact, 'developing' the underlying PTFE orientation layer.

Table 1, finally, gives a sample of the variety of species that were found to form highly oriented films on the PTFE layers.

It is useful to contrast the present orientation technique with previously reported methods that use polymeric substrates. As mentioned above, workers have attempted to induce epitaxial growth of various materials on polymer films oriented by tensile

drawing¹²⁻¹⁴. Note, however, that conventional tensile deformation often yields relatively thick films of only modest, often inhomogeneous orientation, typically with an average angle of mismatch between the draw direction and molecular axis of ~15° or more. Crystalline species grown on these modestly oriented films therefore have only moderate order over limited distances. By contrast, the present ultra-thin PTFE films, and the materials grown on them, display an outstanding degree of orientation, as is shown by Figs 2-4. In addition, unlike our results (see Table 1), relatively few materials have generally been reported to grow in an oriented fashion on films produced by tensile drawing.

In a different, commonly used process, small-molecule liquid crystals are oriented on rubbed or buffed polymer coatings^{16,17}. Presumably the rubbing technique, which involves several steps, induces some orientation of the chains and/or the formation of grooves on the macromolecular substrate surface, which may be responsible for its ability to orient the crystals^{16,17,25}. The present PTFE-deposition technique is much simpler, involving only one step, yields very thin films of outstanding orientation and seems more versatile.

The origin of this remarkable orienting capacity of the PTFE layers is as yet unclear. Nevertheless, the high degree of orientation of the present layers is likely to improve oriented growth of the few species that have previously been reported to show 'epitaxial' interactions with moderately oriented PTFE, such as poly(ε-caprolactone), polyethylene and polyamides¹⁴. But as is evident from Table 1, many more species can be oriented on the present PTFE layers. Their improved orienting capacity and versatility originate in a specific surface topography combined with a favourable crystal structure. The surface, which resembles a nanometre-scale artificial grating, may induce preferential oriented nucleation of the multitude of steps running along the deposition direction, and allow for crystal lattice matching normal to it. In this regard, we note that above 19 °C (where most of the depositions are carried out), the orienting PTFE layers have an unusual crystal structure, with a regular periodicity perpendicular to the chains of ~5 Å, which is a fairly common characteristic dimension in solids; and the structure largely lacks order along the chains, which might have restricted oriented growth. □

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Relaxation modes of the contact line of a liquid spreading on a surface

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THE dynamics of spreading of liquids on solid surfaces are important in several practical and industrial processes, such as painting, lubrication and oil recovery in a porous medium. In all these situations, the motion of the 'contact line' at the edge of the advancing fluid is perturbed by the roughness and chemical contamination of the solid, which distort the line and give rise to poorly understood hysteresis effects. To elucidate these effects, one must begin by studying simplified processes, for example the time response to a perturbation of the line on an ideal surface. Here we describe a study of the dynamics of a contact line under controlled conditions of partial wetting, in which we determine the relationship between the characteristic decay time and the wavelength of a perturbation imposed on the line. When relaxation is controlled by the fluid viscosity, there is good agreement with theoretical predictions, but we observe deviations at long wavelengths which we ascribe to gravitational effects that weaken the contact line's elastic response.

The dynamics of the boundary line between solid, liquid and vapour imply a very unusual form for the elastic energy of the line. When the contact line is deformed following a sinusoid of wavelength $2\pi/q$, where q is the wavevector of the perturbation, Joanny and de Gennes¹ have shown that the energy of the system is proportional to $|q|$ and not to $|q|^2$ which is classically expected in the case of strings. This is because a deformation of the contact line perturbs the liquid-vapour interface over a distance q^{-1} . Integration of the capillary energy over this distance gives the $|q|$ dependence. The effect of this dependence on the collective modes of the contact line has been theoretically derived by de Gennes². Ignoring gravity effects in a first approximation, this model leads to the prediction of two regimes involving different hydrodynamic flows. (1) For large wavevectors, a purely viscous regime is obtained by balancing the elastic force and the friction force due to the dissipation in the liquid edge³. One obtains purely damped modes obeying a linear dispersion law, $\omega = cq$, where the characteristic velocity c depends on the surface tension γ and viscosity, η , of the liquid and on the contact angle, θ , as $c \propto \gamma\theta^3/\eta$ in the small contact angle approximation. (2) For smaller wavevectors, the flow in the edge becomes mainly inertial, which leads to propagating modes of frequency ω varying as $q^{3/2}$.

The aim was then to test these predictions by an experimental determination of the nontrivial dispersion relations. In principle, the experiments involve imposing perturbation of variable wavelength or frequency, and measuring the response of the system. The practical realization of the experiments is rather delicate, as described below.

One of the chief problems is that of achieving a homogeneous solid surface, free of defects, so that the dynamics of the triple line are not masked by pinning on chemical or geometrical singularities. An ideal surface produces no hysteresis: the static contact angles measured when the contact line advances (θ_A) or recedes (θ_R) are identical and equal to the equilibrium angle defined by Young's relation. Surfaces that are generally considered as smooth and clean, such as float glass purified by ozonizing, still give rise to pronounced hysteresis with $\theta_A - \theta_R > 10^\circ$. We succeeded in obtaining a reasonably homogeneous surface by the following process, starting with silicon wafers used in microelectronics for which the rugosity is very small ($\sim 5 \text{ \AA}$). To lower the surface energy of the solid and thus achieve

partial wetting by the liquids used, we grafted a dense layer of long aliphatic or fluorinated chains on the surface^{4,5}. The reactants used were octadecyltrichlorosilane and heptadecafluoro-1,1,2,2-tetrahydrodecyltrichlorosilane leading to critical surface tensions⁶ of ~ 21 and $\sim 15 \text{ mN m}^{-1}$ respectively. The degree of hysteresis is then checked by measuring $\theta_A - \theta_R$. On aliphatic chains $\theta_A - \theta_R$ is always less than 2° , whereas on fluorinated chains, where contact angles are larger, $\theta_A - \theta_R$ is 5° – 8° (see Table 1). In these conditions, we have not observed spurious effects such as distortion of the liquid front.

The other main problem was to induce a periodic deformation of the contact line without damaging the solid surface or the liquid. This requirement precludes, for instance, the use of intense electric fields to produce a macroscopic distortion. Our method, based only on capillary effects, is the following: we deposit on the wafer a row of equidistant small liquid droplets of identical diameter. A rectilinear liquid front is then slowly pushed until it comes into contact with the droplets. This results in a continuous quasi-harmonic deformation which then relaxes to an equilibrium position which is a straight line (Fig. 1). This simple process can easily be used for typical sizes varying from 10^{-2} m to $5 \times 10^{-5} \text{ m}$. For large wavelengths ($> 10^{-3} \text{ m}$) the droplets are deposited one by one. For smaller wavelengths we deposit liquid ridges on the solid surface. The ridges are unstable and spontaneously break into a regular array of circular droplets⁷, the sizes and distances between droplets being fixed by

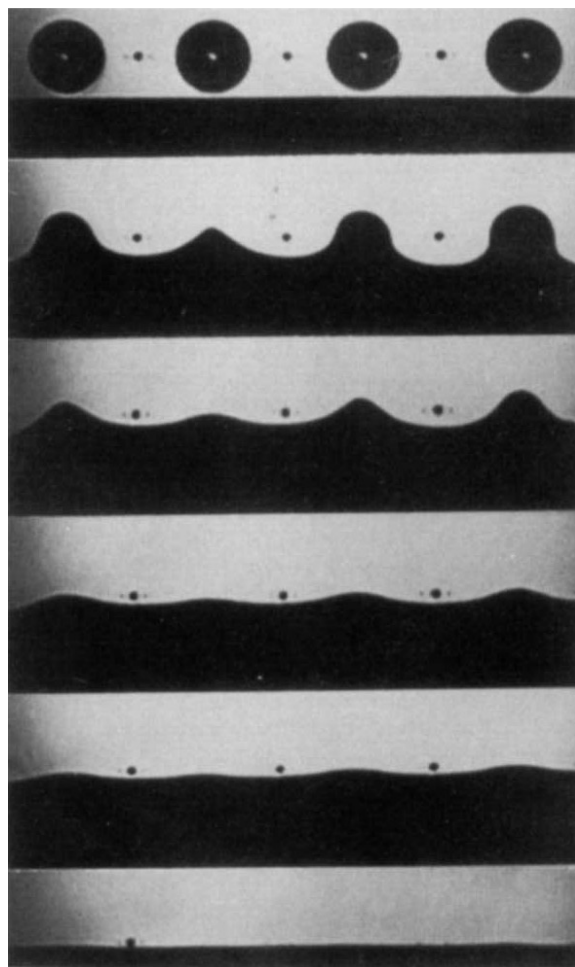


FIG. 1 Photographs of the system at different times during the relaxation process (time increases from top to bottom). The droplets used to induce the deformation of the line were obtained by ridge instability. The small dots between the droplets always appear at the point where the ridge breaks, but they interfere only at the end of the relaxation process, and even then their effect is negligible.

TABLE 1 Characteristics of the liquids and the solid surfaces

Liquid	Surface	η (cP)	γ (mN m ⁻¹)	θ (°)	$\theta_A - \theta_R$ (°)	c (mm s ⁻¹)
47V100	F	96.5	20.9	47.5	5	1.6
47V300	F	291	21.1	47.5	5	0.49
47V500	F	485	21.1	47.5	5	0.33
550	C	132.5	24.5	38	2	0.54
550	F	132.5	24.5	62	8	2.1
641V200	C	212	23.5	28.5	2	0.24
641V200	F	212	23.5	57	8	1.8

The viscosity η and surface tension γ of the silicon oils (Prolabo) were measured by the conventional method; the contact angles θ on the aliphatic (C) and fluorinated (F) chains were measured by the method of ref. 11, or from photographs. The velocity c was deduced from the experimental data.

the width of the initial ridge. Thus, ridges deposited using very thin fibres lead to wavelengths that can be as short as 5×10^{-5} m.

During the relaxation process, the contact line is observed and videotaped through a binocular microscope. The images are then digitalized. As the profile of the perturbation is not a pure sinusoid, we perform its Fourier transform by standard methods. We have found that we always obtain one intense peak corresponding to the imposed wavelength and not more than two other peaks of appreciable amplitude corresponding to higher frequencies. This indicates that our method provides quasi-harmonic perturbation of the line. We measure the amplitude a_q of the different peaks during the relaxation of the line in the regime where the amplitude is small compared with the wavelength, which is the regime in which the theoretical model is valid. In this regime, we check that a_q obeys an exponential law and determine the characteristic time τ_q for each main Fourier component of the spectrum. Repeating this treatment

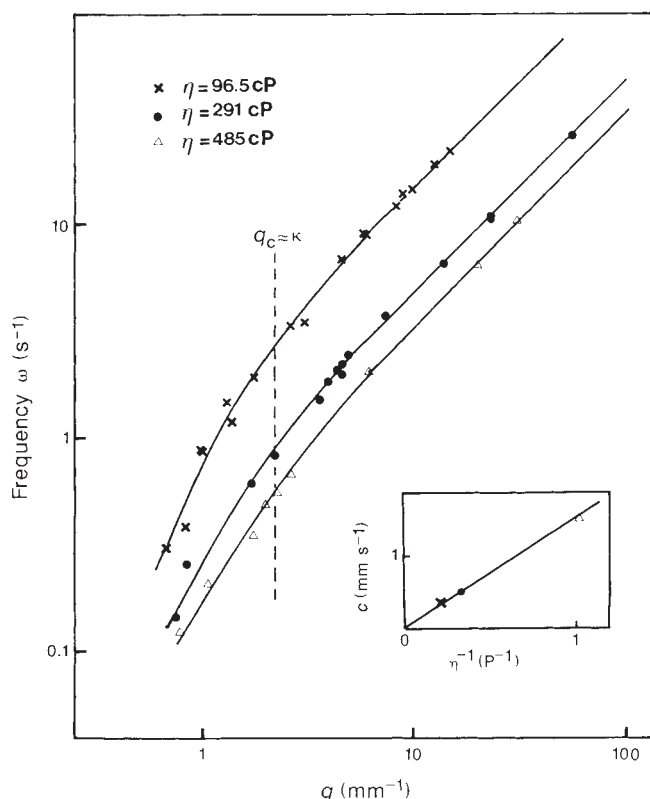


FIG. 2 Experimental dispersion curve for silicon oils 47V100, 47V300 and 47V500 of different viscosities, η , expressed in centipoise, on the same fluorinated wafer. Inset, plot of the velocity c deduced from the values of ω as a function of η^{-1} in the main figure.

for perturbations of initial wavelengths varying from 10^{-2} to 5×10^{-5} m, we can plot the dispersion curve $\omega = f(q)$, where $\omega = 1/\tau$. We have carried out the experiments for different liquids on the two chemically modified silica surfaces in order to study, respectively, the influence of viscosity and of contact angle on the dynamics of the contact line (see Table 1).

Typical data for the experimental dispersion curve are shown in Fig. 2. These were obtained for a single value of the contact angle ($\theta = 47.5^\circ$) while varying the viscosity. Similar results are observed when changing the value of the contact angle for a constant viscosity. All these results show a linear relationship between ω and q , $\omega = cq$, for large values of the wavevector. The characteristic velocity c varies as η^{-1} , as expected (see Fig. 2). The dependence of contact angle is very strong, and is compatible with the θ^3 law predicted by theory. This suggests that the model developed in the small-angle approximation remains valid for a broader range of angles. Analogous observations have been made by Redon *et al.*⁸ in dewetting experiments.

Consequently, in this frequency range, the experimental results agree well with the theoretical predictions about the viscous regime. The deviation from the linear relationship at low q , however, cannot be attributed to the transition to an inertial regime: the experimental crossover occurs for a value q_c of the wavevector which is independent of viscosity and contact angle, and is several orders of magnitude larger than expected for inertial effects². But it is important to note that q_c is of the order of $\kappa = \sqrt{\rho g/\gamma}$ (where ρ is the liquid density and g the acceleration due to gravity), the inverse of the capillary length, which represents the typical scale beyond which gravity effects cannot be neglected. We may then reasonably infer that the low-frequency regime is affected by gravity.

Recently, F. Brochard and co-workers have proposed a more elaborate model including gravity effects (manuscript in preparation). They predict different results depending on the relative values of two characteristic lengths, one of them being κ^{-1} and the other defining the crossover between viscous and inertial effects. In the present experimental conditions (wavevector, viscosity and contact angle), this theory predicts the observation of two different purely damped viscous regimes: (1) when q is larger than κ , gravity effects are negligible so that the viscous regime of de Gennes theory is valid, with the dispersion law $\omega \propto (\gamma \theta^3/\eta)q$; (2) for smaller wavevectors, because of gravity effects, this law is modified, and ω varies as the square of the wavevector: $\omega \propto (\gamma \theta^3/\eta)\kappa^{-1}q^2$. It is worth emphasizing that this last result is valid only when the liquid is spreading horizontally. In the case of capillary rise, the elastic energy is very different^{9,10}, and the relaxation frequency would be expected to reach a finite limit at small wavevector.

This description of the viscous modes seems to agree well with our experimental data: as previously underlined, the linear dispersion law is obtained at high frequency with the correct dependence of c on η and θ ; the crossover occurs for $q \approx \kappa$, and the data obtained at the lowest wavevector tend reasonably to a q^2 dependence. To test this second dispersion law, it would be interesting to extend our experiments to lower q by producing larger wavelengths of distortion (several centimetres), but this would require very large homogeneous surface free of defects, which are difficult to attain. In the future we hope to explore the propagating inertial modes with liquids of low viscosity. It would also be interesting to test the difference of behaviour in the capillary-rise geometry. □

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Palaeobotanical evidence for a June 'impact winter' at the Cretaceous/Tertiary boundary

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A LARGE bolide impact, such as that thought to have occurred at the Cretaceous/Tertiary (K/T) boundary, should produce large amounts of light-attenuating debris, thereby causing an 'impact winter'^{1–3}. Because of thermal buffering in the oceans, evidence for a brief (1–2 months^{2–4}) impact winter would be found only in terrestrial environments. Aquatic leaves in the K/T boundary section near Teapot Dome, Wyoming, preserve structural deformation that can be duplicated experimentally in extant aquatic leaves by freezing. Reproductive stages reached by the fossil aquatic plants at the time of death suggest that freezing took place in approximately early June. Both the existence of the structurally deformed plants and the high abundance of fern spores occur in a horizon containing sparse impact debris, but below the horizon containing abundant impact debris; I therefore suggest that the lower horizon represents debris and effects from a large, distant bolide impact, and the upper horizon represents a small, nearby bolide impact.

An impact winter could produce widespread extinctions; such extinctions could indicate that a catastrophe had occurred but not the nature of the catastrophe. Tschudy⁵ inferred widespread ecological disruption (but not necessarily extinction) at the K/T boundary in the western interior of North America from the fern spike, which is a sudden and marked increase in abundance of a single type of fern spore in a given K/T boundary section. Tschudy considered this to be analogous to the early successional vegetation dominated by ferns following mass-kill from large volcanic eruptions such as Krakatoa⁶. Mass-kill from volcanic eruptions, however, typically results from winds and heat generated by the eruption⁷, and, except within a few hundred kilometres of the impact crater, the K/T bolide would not cause immediate mass-kill by direct transfer of thermal or kinetic energy to land organisms. Tschudy nevertheless inferred that some physical consequence of the K/T bolide impact resulted in the fern spike, because in sections analysed (mostly in the Raton Basin of New Mexico and Colorado), the fern spike occurred immediately above the impact layer⁸, which contains abundant, large, shock-metamorphosed minerals and is enriched in iridium. The fern spike has also been recorded above the impact layer in eastern Montana⁹ and Saskatchewan¹⁰ but is not evident in central Alberta¹¹.

An apparently anomalous report¹² based on the K/T boundary section on Dogie Creek in eastern Wyoming placed the fern spike below the impact layer. At Dogie Creek, the fern spike is in the boundary claystone, an interval that had previously (especially in the Raton Basin) yielded few palynomorphs. From a Raton Basin sample of boundary claystone supplied by G. A. Izett from the Madrid East site⁸, I obtained abundant, well preserved palynomorphs, 91% of which are one type of fern spore. Near Teapot Dome, 100 km west of the Dogie Creek site, the fern spike also occurs below the impact layer (Fig. 1; ref. 13). It seems, therefore, that the palynological

evidence of considerable ecological disruption is present in a horizon preceding the horizon that has physical evidence of early fallout from bolide impact. But detailed palaeobotanical analysis of the Teapot Dome section (Fig. 2), which is the only western interior section that preserves identifiable plant megafossils through the boundary interval, resolves this apparent anomaly and may indicate the intensity and timing of environmental changes at the K/T boundary.

The Teapot Dome site was a lily pond, as shown by the abundance and diversity of structures of aquatic plants (Figs 2 and 3); extant relatives of some of the fossils are confined to ponds less than 2 m deep¹⁴. Leaves of aquatics such as the pond lily (*Paranymphaea*, allied to the extant *Nuphar* of Nymphaeaceae) and lotus (*Nelumbites*, allied to the extant *Nelumbo* of Nelumbonaceae) are typically not preserved as fossils because of very low sedimentation rates in lily ponds and the biological degradation and fragmentation of such leaves while still floating on the water surface; the Teapot Dome site, however, preserves thousands of these leaves in fine-grained sediments. The leaves are preserved, moreover, in a light-coloured clay, very unlike the carbonaceous mudstone which was the characteristic deposit in this lily pond before the boundary interval. The *Nuphar*-like rhizomes and *Nelumbo*-like growing tips (with roots) are fragmented and do not occur in their original growth positions, which could suggest high-energy transport by rapid water flow, but such transport is negated by the fine-grained sediments. The aquatic plants were suddenly killed, their below-surface roots and rhizomes were pulled off the pond bottom, and, after a period of decay, the aquatic remains were covered by an influx of fine-grained debris. The *Paranymphaea* leaves, which may have been emergent as in many extant *Nuphar*, are physiognomically like typical latest Cretaceous (but unlike early Palaeocene) leaves. I infer that the aquatic plants preserved at Teapot Dome were alive during the latest Cretaceous.

The fossils, moreover, include immature pollen of apparent Nelumbonaceae and pollen of Nymphaeaceae. Both pollen types occur in antherial masses, suggesting that they derive from flowers at or near the site of deposition. Although the pollen types are not identical to pollen of extant *Nelumbo* and *Nuphar*, the association of only two taxa of megafossils with antherial

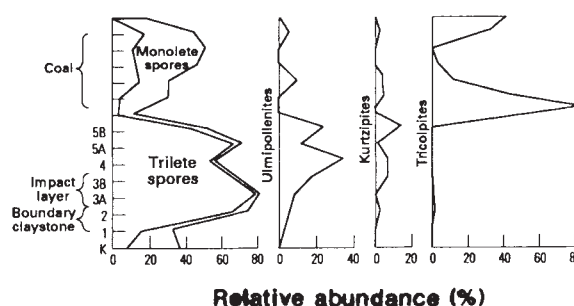


FIG. 1 Palynomorph diagram of the Teapot Dome boundary interval. The sample from bed 1 contains the typically Cretaceous *Proteacidites*. The high relative abundance of ferns (fern spike) occurs in the clasts of bed 2 below beds 3 and 4 ('impact layer'); nelumbonaceous pollen is abundant in bed 2. Relative (but not necessarily absolute) abundance of fern spores decreases as a few kinds of probable deciduous plants (*Kurtzipites*, *Ulmipollenites*, *Tricolpites*) increase. The fern spike of the clasts of bed 2 is composed mostly of a single type of fern spore, whereas the fern abundance in the upper part of the coal is composed of at least five spore types. A very few kinds of tropical ferns can grow new shoots from rhizomes and spore in 3–4 weeks (R. Foster, personal communication), and this probably resulted in the fern spike; the second fern abundance may represent sporing by plants that had grown from spores (see also ref. 7) after the impact winter ended. Ferns show no significant increase and deciduous plants continue flowering after deposition of the impact layer, suggesting that an impact winter did not result from the second impact.

masses of two pollen types argues for the megafossils and microfossils being derived from the same plants. If the plants were still flowering and the *Nuphar*-like seeds were freshly produced, the aquatics were killed before the end of the growing season.

The leaf cuticles found in clasts of bed 2 and in beds 3–5 have pronounced irregular folds, which are absent in cuticles of bed 1 and the matrix of bed 2. Leaves of extant lotus, *Nelumbo nucifera*, were frozen for 2–7 weeks and show the same type of irregular folds as the fossils, but unfrozen control leaves and unfrozen dried leaves do not. Folds in the modern leaves were

TABLE 1 Suggested sequence of events represented in Teapot Dome K/T boundary interval

Bed 5	Finer debris from second impact falls on, and is washed into, pond; partially decayed, heavy <i>Nelumbites</i> rhizomes (Fig. 3d) deposited at base; decaying <i>Paranymphaea</i> leaves (Fig. 3i), pollen (Fig. 3e) and seeds (Fig. 3k), and <i>Nelumbites</i> leaves (Fig. 3j) and growing tips (Fig. 3h) randomly deposited; deciduous plants continue flowering; mean daily temperature 30 °C.
Bed 4	Little sedimentation; decaying debris falling to bottom is compacted, forming carbonaceous sediment.
Bed 3	Shock-metamorphosed minerals from second impact arrive; some decaying leaves deposited, forming layering; more deciduous plants begin flowering.
Bed 2	Mean daily temperature rises to 20 °C; fallout debris continues to be washed into pond; a single kind of fern reaches sporing stage; a few kinds of deciduous plants break dormancy and flower; mean daily temperature rises to 25–30 °C; spores and pollen fall on decaying leaves still covered with some fallout debris, which concentrates (with pollen and spores) as pods on cup-shaped <i>Nelumbites</i> leaves (Fig. 3j) and hardens in sun; some leaves decay further, releasing pods that, with some debris-filled petioles, fall on, and are incorporated in, upper part of bed 2; second bolide strikes; microtektite shower.
Bed 1	Mean daily temperature rises above 0 °C; rains begin; influx of water into pond area buoys solid ice upward, pulling growing tips of <i>Paranymphaea</i> rhizomes and <i>Nelumbites</i> off pond bottom and from upper part of Cretaceous pond sediment; ice melts rapidly, and fallout debris falls to bottom of pond; sediments rapidly wash in, unlayered, mostly comprising impact fallout but including some debris on land surface at time of impact (including remains of Cretaceous plants such as cuticles of ' <i>Artocarpus</i> ' dissecta and pollen of <i>Proteacidites</i>); a single kind of fern sends up new shoots from rhizomes.
Between latest Cretaceous carbonaceous shale and bed 1	Within a few days, mean daily temperature falls to –5 to –10 °C; pond freezes over (possibly to bottom); finer impact debris arrives after freezing, falling on ice and surrounding land; aquatic plants die, some frozen into ice and some falling on ice; many terrestrial plants, especially broad-leaved evergreens, die or are killed back, but some deciduous plants re-enter dormancy; no deposition on pond bottom.
Latest Cretaceous	Mean daily temperature ~19 °C; <i>Paranymphaea</i> flowering and fruiting, <i>Nelumbites</i> flower buds still unopened; pond also covered with abundant floating water-fern (<i>Azolla</i>); first bolide strikes, and microtektites arrive.

formed by formation of extracellular ice between outermost cells and the cuticle, followed by plastic expansion of the cuticle to accommodate this ice.

At the time of freezing at Teapot Dome, *Paranymphaea* had leafed out, bloomed and produced seeds. In contrast, although the more abundant *Nelumbites* had leafed out, it had not produced fruits, and the flowers were still immature. Where they coexist today, *Nuphar* blooms and fruits before *Nelumbo*, which is a strong parallel to the relative reproductive timing of the Teapot Dome aquatics. Analysis of meteorological data for the present geographic range of *Nelumbo* indicates that mature blooms develop about 2 months after mean temperature reaches 16 °C. Latest Cretaceous temperatures inferred for eastern Wyoming¹⁵ indicate that a mean temperature of 16 °C would occur in late April; Nelumbonaceae would then bloom in late June, and thus the freezing would have been in early June. The Teapot Dome *Nelumbites* and *Paranymphaea* are separated in time from their closest modern relatives by 66 Myr and thus may (but not necessarily) have evolved physiologically in this interval; this possibility could introduce an error of a few weeks in the suggested time of freezing.

Abundant large, shock-metamorphosed quartz grains and abundant spherules (possible microtektites) were deposited after mass-kill by freezing of the aquatics at Teapot Dome and the early stage of recovery indicated by the fern spike. The boundary claystone contains rare, small, shocked quartz grains (B. F. Bohor, personal communication), as in marine K/T boundary sections¹⁶, above-background iridium, and some spherules (possible microtektites). I suggest that the boundary claystone represents debris from a large bolide that struck the Earth in

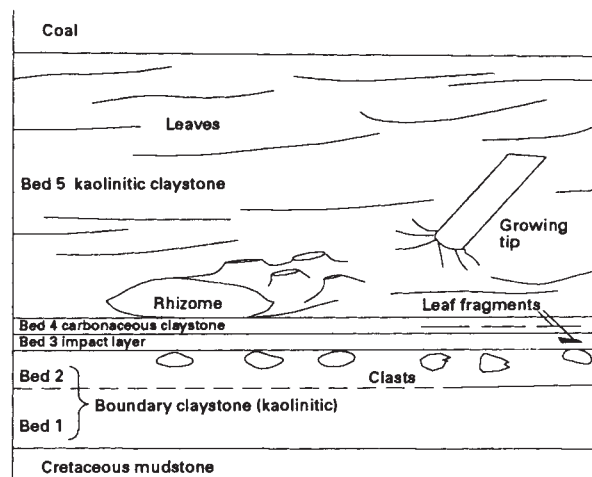
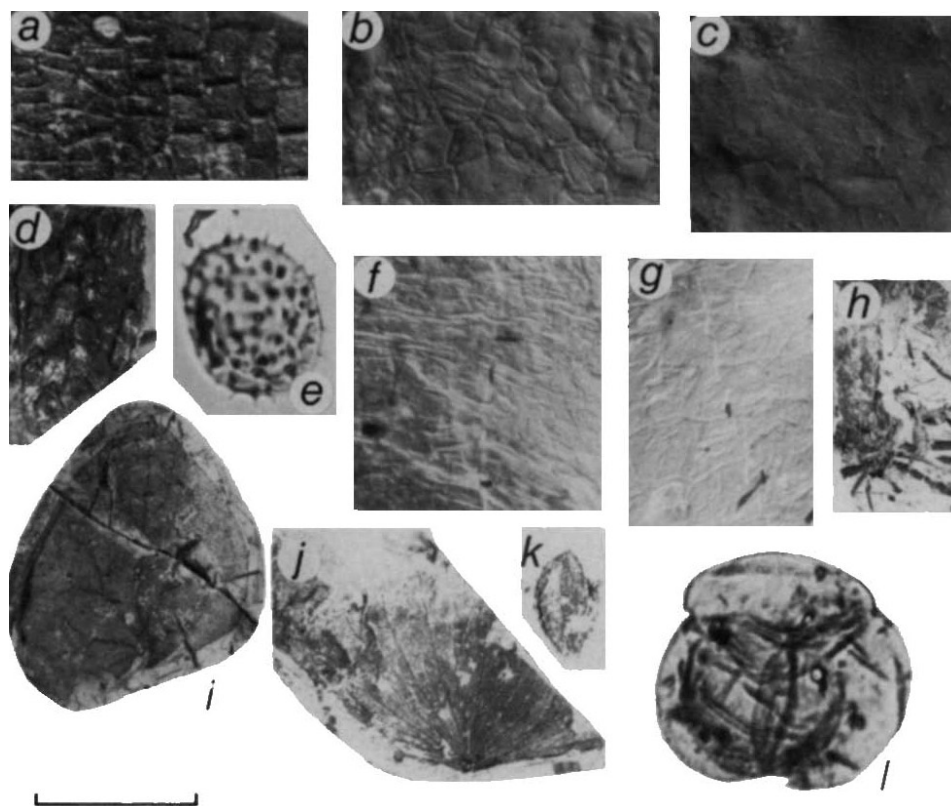


FIG. 2 Diagram of the boundary interval at Teapot Dome shown at natural size. For purposes of analysis, five beds were designated; contacts between the beds are gradational on a scale too small to be depicted. Maximum iridium abundance is in the upper part of bed 2; this concentration may be related⁸ to post-depositional movement of iridium toward the carbonaceous-rich beds 2 and 3. The concentration of iridium in bed 3 is 22 p.p.b. No evidence (for example, the presence of fusinite) was seen for wildfires in the heavy or fine fractions of the palynomorph samples from the boundary interval (compare with ref. 21, who considers the effects of possible wildfires to have been limited). Some clasts in bed 2 represent partially decayed petioles filled with claystone of texture different from the surrounding matrix; when viewed from the top, the matrix around the clasts is concentrically arranged, as if the clasts fell vertically. The clasts contain structurally deformed cuticles and abundant fern spores, but the matrix of bed 2 does not. Spherules, mostly of goyazite but some of clay, occur abundantly in bed 2 and sparingly in the lower part of bed 1; these have been considered authigenic⁸ and also have been considered as altered microtektites. The second suggestion is probably valid in light of the discovery of pristine and altered microtektites in the Haitian boundary section^{22,23}.

FIG. 3 Fossils from the K/T boundary section near Teapot Dome. The micrographs are to different scales, and the length represented by the scale bar (bottom left) is given in parentheses for each image. *a*, Cuticle from bed 1; note absence of irregular folds. (Scale bar 75 μ m.) *b*, *f*, Cuticles from clasts of bed 2; note pronounced, irregular folds (75 μ m). *c*, *g*, Leaves of extant lotus (*Nelumbo nucifera*) that were frozen in water at -10°C for 4 weeks (75 μ m). *d*, Piece of a *Nuphar*-like rhizome with leaf scars, from base of bed 5; the rhizome was broken, detached from leaves, and partially decayed before burial (3 cm). *e*, Mature *Nuphar*-like pollen grain from bed 3 (40 μ m). *h*, Growing tips of *Nelumbonaceae* with attached roots from bed 5; the growing tips were detached from both rhizome and leaves and were partially decayed before burial (3 cm). *i*, Leaf of *Paranymphaea crassifolia* from bed 5. Note small size and rounded apex; leaves of this species from the early Palaeocene are 14–15 cm wide on average and have acute apices (3 cm). *j*, Leaf of *Nelumbites* from bed 5; the cup-shaped leaf was partially decayed before burial (3 cm). *k*, *Nuphar*-like seed from bed 5; the aril, which is a seed coat on freshly produced seeds and assists in floating, is present on most specimens (7.5 mm). *l*, Tetrad of *Nelumbo*-like pollen from clasts of bed 2; in extant *Nelumbo*, tetrads occur in young (immature) pollen²⁴ (50 μ m).



distant, quartz-poor terrain, and that the impact layer mostly represents debris from a bolide that landed in quartz-rich terrain near the western interior.

Table 1 gives the proposed sequence of events. The time between the initiation of the impact winter and the deposition of bed 5 may have been as brief as 3 months and probably was not longer than 4 months. The impact winter lasted for at least 1–2 weeks, the time necessary for fallout debris to occur in both hemispheres³; on the other hand, the survival of large, aquatic ectotherms indicates an impact winter of less than 2 months⁴. After the attenuation of solar energy ended, the greenhouse effect would occur immediately¹⁷ and promote plant growth and some ferns sporing. Large, tropical aquatic plants substantially decay in a few weeks or months¹⁸, and in my experiments with modern *Nelumbo* leaves frozen and then left in pond water at a temperature of 30°C , the leaves reached a stage of decay as in the Teapot Dome leaves in ~ 8 weeks. The second impact, the debris from which buried the leaves, probably occurred ~ 8 weeks after the impact winter ended and ~ 10 – 16 weeks after the first impact.

In this proposed scheme, I do not envisage a landscape totally denuded of vegetation (except for ferns) at Teapot Dome following the impact winter. Deciduous trees and shrubs were an important but subdominant element in the Late Cretaceous vegetation of eastern Wyoming⁴, and some flowered within a few weeks or months after the impact winter ended. Some cold-tolerant broad-leaved evergreens survived. Leaves of a fan palm occur ~ 20 cm above bed 5; the extant Mediterranean fan palm (*Chamaerops*) can survive temperatures of -14°C and can produce new shoots from basal clumps¹⁹.

The impact winter had little effect on the land biota at high

latitudes of the Southern Hemisphere²⁰, which were almost at mid-winter. In the Raton Basin, deciduous plants were uncommon in the latest Cretaceous⁴, and the persistence of the fern spike above the impact layer reflects the scarcity of deciduous plants and slow recovery of evergreen plants. In Alberta, deciduous plants dominated in the latest Cretaceous⁴; in the palynofloras, many deciduous taxa survived and shared dominance with the ferns. The impact winter, although significant in the northern hemisphere, may have had less effect on the land biota than did the following, long-lasting wet greenhouse¹⁵. □

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An invasion percolation model of drainage network evolution

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STREAM networks evolve by headward growth and branching away from escarpments such as rift margins. The structure of these networks and their topographic relief are known to be fractal¹⁻³, but no model so far has been able to generate the observed scaling properties. Here I present a statistical model of network growth in which stream heads branch and propagate at a rate that depends only on the local strength of the substrate. This model corresponds to the process of invasion percolation⁴, with the added requirement of self-avoidance; it is a self-organized critical system⁵ with properties similar to those of standard percolation models⁶. A description based on self-avoiding invasion percolation reproduces the known scaling behaviour of stream networks, and may provide a valuable tool for delineation of drainage patterns from digital topographic data sets^{7,8}.

Many stochastic models of river network evolution have been proposed, ranging from the early work of Leopold and Langbein⁹ on self-avoiding random walks, to the computer simulations of Howard¹⁰ and the aggregation model of Scheidegger¹¹. The present study was prompted by the suggestion of Mandelbrot¹ that rivers are fractal. The evidence for this lies in the power-law relation between principal stream length and drainage basin area^{12,13}. Hack¹⁴ discovered that the scaling exponent d_H is ≈ 0.6 and not a simple square-root relation as one might expect; such behaviour holds over a remarkable range of scales

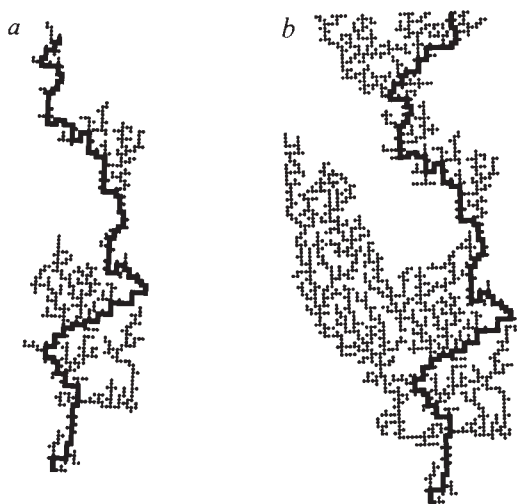


FIG. 1 The development of a self-avoiding invasion percolation cluster, shown at two stages of growth. On average the whole cluster has a fractal dimension of 1.896 and the fractal dimension of the shortest path, marked in heavy type, remains at 1.130 throughout.

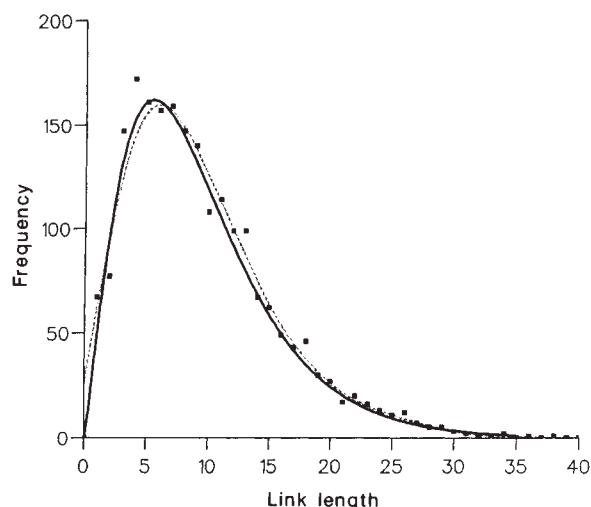


FIG. 2 Distribution of interior-link lengths for the self-avoiding invasion percolation model, from 20 runs on a 256×256 lattice. The bold line is a fit to a gamma function, and the dashed line a smoothed fit to the data points.

from laboratory experiments to basins almost the size of the Amazon. The Hack relation may be rewritten:

$$L_p \sim L^{d_{\min}} \quad d_H = d_{\min}/2 \quad (1)$$

where L is drainage basin length, L_p is principal stream length, and $d_{\min}$ is the fractal dimension of the principal stream. A reliable estimate¹⁵ for d_H is 0.568.

In formulating a model of headward growth and branching, we must consider noise in the substrate. Variation in the lithology, structure, diagenesis, groundwater hydrology, soil development, vegetation and topography leads to considerable variation in substrate strength. The manner in which this noise is incorporated into any model of drainage evolution is crucial to the resulting network geometry. Consider a simple model of headward growth of streams away from a rift margin¹⁶ in which stream heads initiate and propagate away from the uplifted escarpment. Bank failure and stream propagation may occur anywhere on the perimeter of the stream network; if the substrate strength varies, however, the probability of failure is far higher at some points on the perimeter than at others. If we make the simplification that at any particular time the next point to fail will be the weakest, the model of headward growth and branching becomes invasion percolation⁴. In invasion percolation, a regular lattice is set up with random strengths assigned to bonds between initially empty sites. The type and dimension of the lattice are not important, nor is the exact distribution of bond strengths. Initially, one seed site is occupied; growth occurs by forming a link along the weakest bond from this seed, and by occupying the linked site. Further growth continues by a similar process around the perimeter of the cluster. The system rapidly self-organizes to a stage where a restricted range of bond strengths is chosen, and the cluster grows indefinitely. The cluster shows the same fractal scaling as standard lattice percolation clusters⁶ and as continuum or off-lattice percolation clusters¹⁷.

Streams rarely bifurcate downstream, so a non-looping¹⁸ condition is imposed on growing clusters. This is straightforward to implement: no site may be invaded if any of its neighbour sites except the source site are already occupied. This is a new model which I will call self-avoiding invasion percolation (Fig. 1). Models such as lattice percolation, invasion percolation and self-avoiding invasion percolation all belong to the same universality class⁶ and their scaling properties are identical. Hence we can write¹⁹ the distance L_p along a model principal stream as

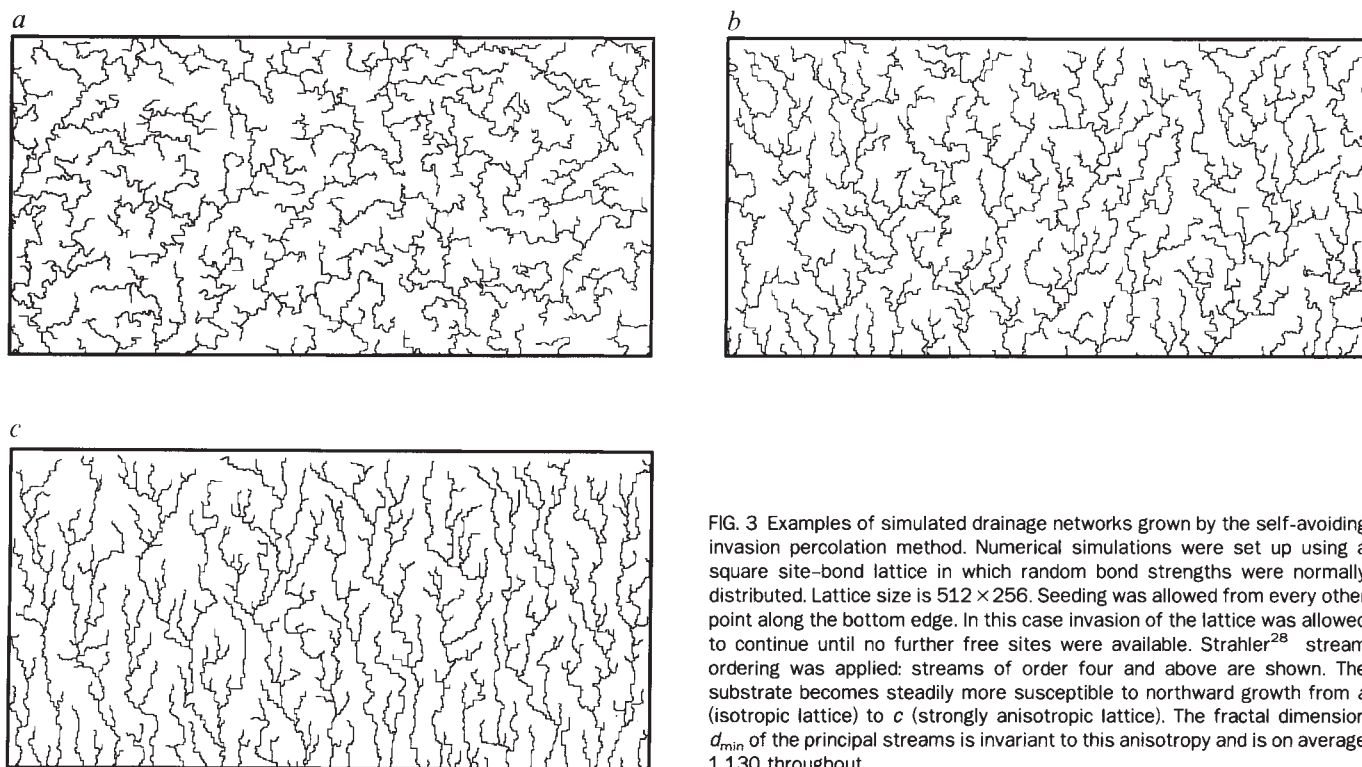


FIG. 3 Examples of simulated drainage networks grown by the self-avoiding invasion percolation method. Numerical simulations were set up using a square site-bond lattice in which random bond strengths were normally distributed. Lattice size is 512×256 . Seeding was allowed from every other point along the bottom edge. In this case invasion of the lattice was allowed to continue until no further free sites were available. Strahler²⁸ stream ordering was applied: streams of order four and above are shown. The substrate becomes steadily more susceptible to northward growth from *a* (isotropic lattice) to *c* (strongly anisotropic lattice). The fractal dimension $d_{\min}$ of the principal streams is invariant to this anisotropy and is on average 1.130 throughout.

a function of cluster length L as $L_p \sim L^{d_{\min}}$ where $d_{\min} = 1.130$, the scaling exponent for the cluster backbone¹⁹, and where L is equivalent to drainage basin length. We predict that $d_H = d_{\min}/2 = 0.565$. This result is identical to equation (1) and is consistent with the observed¹⁵ value of $d_H \approx 0.568$. It is striking that the model not only predicts fractal scaling of streams but also derives the observed dimension of those streams so accurately. The self-avoiding invasion percolation model predicts other fractal properties for drainage networks; these have been shown recently²⁰ to be closely related to order ratios for the streams. For example, the stream networks will obey:

$$d_f = \frac{2 \log R_B}{\log R_A} = 1.896 \quad (2)$$

$$d_l = \frac{\log R_B}{\log R_L} = 1.678 \quad (3)$$

where $d_l = d_f/d_{\min}$; d_f is the fractal dimension of the whole network, d_l is the intrinsic dimension¹⁷ and R_B , R_L and R_A are the bifurcation, length and area ratios, respectively. Recently obtained values for natural stream networks include $d_f \approx 1.90$, $d_{\min} \approx 1.12$ and $d_l \approx 1.65$ from order ratios^{13,21}, and $d_f \approx 1.90$, $d_{\min} \approx 1.16$ from direct (box-counting) fractal measurement¹³. These data are remarkably consistent with the predicted values above. Furthermore, the interior links (the shortest distances between stream junctions) of the model networks are gamma-distributed (Fig. 2), as has been observed for natural systems²².

An examination of previous models of drainage network evolution shows that few predicted fractal scaling. For example, Howard's computer simulations¹⁰ were essentially Eden tree growth²³. This is a kinetic model where new sites are added to the perimeter of the cluster at random; in our model this is equivalent to randomly reassigning bond strengths to the lattice at each step. In this system there is no 'history' to the distribution of bond strengths around the growing cluster. The resulting networks are topologically random²⁴ and Euclidean, with $d_{\min} = 1.0$ and $d_f = d_l = 2.0$. The 'top-down' model of Scheidegger²⁵, in which flow paths coalesce randomly downslope, shows similar

scaling. Diffusion-limited aggregation has also been suggested as a suitable model for drainage evolution¹⁶, but fails because the scaling exponent of shortest paths $d_{\min}$ on a diffusion-limited aggregate²⁶ is 1.0. In addition, for such clusters the probability of growth is proportional to the local gradient of the potential field²⁷. This potential field ϕ is constrained in the model to obey Laplace's equation, $\nabla^2 \phi = 0$, and to be subject to considerable noise. It is not at all clear what potential field determines substrate failure, or whether any such field should satisfy Laplace's equation.

The self-avoiding invasion percolation model can account for substrate sensitivity to local topography without any change in the scaling behaviour of the drainage networks. Similarly, a strong regional tilt may be modelled without affecting the fractal properties of the streams. Figure 3 shows the effect of biasing failure probability in one particular direction: stream networks range from isotropic (*a*) to anisotropic and dendritic (*c*) as the bias is increased, but the fractal scaling of the networks remains invariant. In a percolation model, drainage propagation, controlled by substrate strength, and drainage delineation, controlled by local gradient, are equivalent. This suggests a simple method for drainage delineation in which lattice bond values are calculated by differencing a grid of topographic heights. Streams are then traced out upstream, rather than downstream as is usual, by a self-avoiding growth mechanism. The fractal geometries of stream networks are more likely to be preserved by this method than by existing algorithms.

In conclusion, it seems likely that the dynamical link between surface flow and topography is under-pinned by a self-avoiding invasion percolation process, and that the reason for fractal scaling both of topography^{2,3} and drainage networks^{1,12,13} may lie in this percolation property of geomorphology. □

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Sources of sedimentary lipids deduced from stable carbon-isotope analyses of individual compounds

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COMPOUND-specific isotope analysis by gas chromatography combined with isotope-ratio mass spectrometry (GC-IRMS)^{1,2} provides a new tool with which to study the carbon cycle at the molecular scale³. Previous studies^{2,4} using this technique have been concerned with oceanic systems. Here we demonstrate that the potential for elucidating terrestrial sedimentary processes is equally important. By comparing the carbon isotope ratios ($\delta^{13}\text{C}$) of individual *n*-alkanes from the leaves of lakeside trees with those from the lake sediments, we are able to discriminate between the diverse sources of the sedimentary carbon. The leaf-wax *n*-alkanes show a large inter-species $\delta^{13}\text{C}$ variation of –30.1 to –38.7%, which may be the result of genetic differences in plant adaptation and physiology. Values of –30.1 to –35.9% were obtained for the corresponding *n*-alkanes extracted from the lake sediments, indicating that they derive from a mixed input of deciduous leaf waxes. Shorter-chain lipids in the sediments had $\delta^{13}\text{C}$ values of –20 to –22%, implying that these originate from a different (probably algal) source. Information of this sort goes beyond that which can be deduced from bulk isotope or biomarker analyses alone.

Measurement of carbon isotope ratios in bulk organic matter has become widely used. For example, carbon isotope discrimination in relation to plant physiology has been extensively investigated^{5–10} and variations in $\delta^{13}\text{C}$ values in terrestrial C3 plant tissue have been attributed to factors that affect the internal concentration of carbon dioxide in the leaf⁸, such as light irradiation⁹, efficiency of water use⁷ and stomatal resistance¹⁰.

Comparison of $\delta^{13}\text{C}$ data for bulk plant biomass with values for organic carbon from sedimentary cores has assisted in the reconstruction of ancient biogeochemical processes¹¹. GC-IRMS¹ now permits this comparison to be carried out at the

molecular level, resulting in a much more detailed picture of the biogeochemistry of sedimentary ecosystems^{2,4}. Earlier studies have dealt with ancient aquatic systems, in which the fractionation of carbon isotopes is related to the concentration of dissolved bicarbonate in water⁴. In terrestrial plants, however, $\delta^{13}\text{C}$ is related to CO_2 gas-exchange factors operating in air^{6,7}. The $\delta^{13}\text{C}$ information currently available for individual compounds in terrestrial plants or fresh-water sedimentary organic matter is very limited and based on lengthy separation techniques, with $\delta^{13}\text{C}$ analyses by conventional combustion IRMS^{12,13}. With GC-IRMS, such analyses can be carried out simply and routinely^{2,4}, allowing stable isotope research to be applied in many diverse disciplines³.

We sampled six deciduous tree species growing close to each other around Ellesmere lake¹⁴ (United Kingdom) in November 1989. Three samples of sediment from a short core taken from the lake were examined for comparison. The sampling, extraction methods and detailed lipid distributions are described by Rieley *et al.*¹⁵. The $\delta^{13}\text{C}$ measurements reported here for the *n*-alkane fractions were obtained using a VG Isochrom II GC-IRMS; a representative ion-current chromatogram at relative molecular mass $M_r = 44$ is given in Fig. 1.

The $\delta^{13}\text{C}$ results for the individual *n*-alkanes and *n*-alcohols are given in Table 1. Values of $\delta^{13}\text{C}$ for the *n*-alkanes C_{25} to C_{33} in the sediment samples vary from –30.1 to –35.9%, which are consistent with bulk values for higher plants following the C3 metabolic pathway¹⁶. These values are much more negative than those of other aliphatic lipids assigned to an autochthonous (algal) source in the same sedimentary system, which had $\delta^{13}\text{C} = -22.7$ and –23.8% for the C_{14} and C_{16} *n*-alcohols¹⁷, respectively. Furthermore, the mean $\delta^{13}\text{C}$ values for the leaf-wax *n*-alkanes in the same carbon-number range vary from –30.1 to –38.4%. Thus, the sedimentary *n*-alkanes and *n*-alkanols in the range C_{25} to C_{33} can be attributed primarily to the input of leaves from deciduous trees fringing the lake. The advantage of GC-IRMS over conventional isotope analyses of bulk organic carbon is illustrated by this discrimination between compounds originating from fresh-water algae and from terrestrial plants. In contrast, the two sediment samples taken from 0–5 cm and 10–15 cm in Ellesmere had $\delta^{13}\text{C}$ values for total organic carbon that were identical (–27.4%), representing an averaging of all possible sources and of the products of diagenesis. The identical bulk $\delta^{13}\text{C}$ values could be interpreted as indicating a constant source for the sedimentary carbon, whereas the GC-IRMS

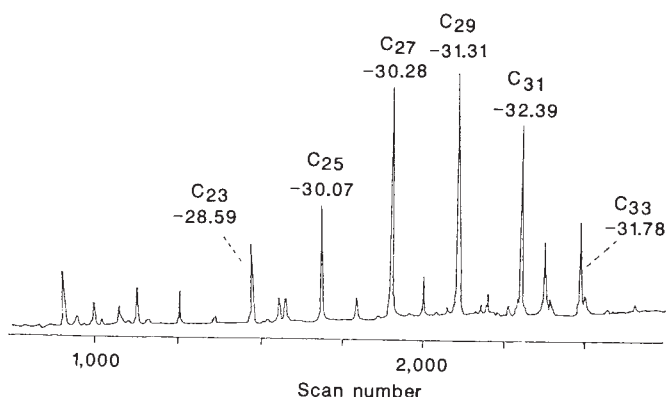


FIG. 1. Hydrocarbon fraction from Ellesmere sediment at 10 to 40 cm depth. This is the relative molecular mass $M_r = 44$ ion-current chromatogram, obtained from the continuous analysis of the combusted GC effluent by a mass spectrometer¹. The *n*-alkane carbon numbers are given above $\delta^{13}\text{C}$ values for each peak analysed. GC column: 25 m $\times$ 0.32 mm internal diameter fused silicone (CP SIL 5CB); helium carrier gas; temperature programme, increasing from 80–300° at a rate of $\sim 4^\circ \text{ min}^{-1}$, then isothermal at 300° for 20 min. $\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3\text{‰}$ PDB, where $R = {}^{13}\text{C}/{}^{12}\text{C}$ and $R_{\text{standard}} = 0.0112372$ based on Pee Dee belemnite.

TABLE 1 GC-IRMS measurements of $\delta^{13}\text{C}$ for *n*-alkanes and *n*-alcohols in Ellesmere leaf waxes and lake sediments

Tree species	<i>n</i> -alkane carbon number						Mean
	C ₂₅	C ₂₇	C ₂₉	C ₃₁	C ₃₃		
<i>Fagus sylvatica</i> L.	—	−30.1 (0.2)	—	—	—		−30.1
<i>Alnus glutinosa</i> (L.) Gaertn.	—	−33.1 (0.4)	−32.0 (0.2)	—	—		−32.6 (0.8)
<i>Salix alba</i> L.	−35.2 (0.6)	−34.5 (0.2)	−35.2 (0.2)	—	—		−35.0 (0.4)
<i>Quercus robur</i> L.	−36.0 (0.5)	−35.0 (0.3)	−35.9 (0.04)	−36.3 (0.4)	—		−35.8 (0.6)
<i>Acer pseudoplatanus</i> L.	−32.1 (0.2)	−34.9 (0.1)	−37.3 (0.1)	−34.9 (0.1)	−33.2 (0.3)		−34.5 (2.0)
<i>Aesculus hippocastanum</i> L.	—	−38.0 (0.1)	−38.7 (0.3)	—	—		−38.4 (0.5)
Sediment samples							
0–5 cm	−32.0 (0.4)	−32.3 (0.2)	−34.0 (0.4)	−35.9 (0.5)	−35.7 (—)		−34.0 (1.8)
10–15 cm	−33.5 (1.8)	−31.6 (0.5)	−32.2 (0.2)	−33.4 (0.1)	—		−32.7 (0.9)
10–40 cm	−30.1 (0.1)	−30.3 (0.2)	−31.3 (0.2)	−32.4 (0.2)	−31.7 (0.2)		−32.2 (1.0)
ELS 10–40 cm	<i>n</i> -alcohol carbon number						C ₃₀
	C ₁₄	C ₁₆	C ₂₂	C ₂₄	C ₂₆	C ₂₈	
ELS 10–40 cm	−22.7	−23.6	−32.6	−32.1	−33.4	−32.4	−31.4

All values are ‰ PDB; values in parentheses are σ_{n-1} . All GC-IRMS runs were repeated up to five times, although GC-IRMS runs with low *n*-alkane concentrations were disregarded. Mean values are arithmetic averages of the individual leaf-wax homologue means.

results show a clear difference in their compound-specific $\delta^{13}\text{C}$ values, indicating a difference in input over time. If only the bulk value is used, much useful information is lost.

The considerable range of 8‰ between the tree species is significant, given that the samples were all taken from within 500 m of each other in open woodland, growing upon the same soil, with presumably similar supplies of moisture and nutrient. Such a wide range has only previously been reported for bulk plant tissue sampled across different seasons and between distinct vegetation types¹⁸. This range (−30.1 to −38.4‰) for the leaf-wax *n*-alkanes is therefore presumably caused mainly by genetic variation in C_i (refs 6, 7), rather than differences in physical factors such as light irradiation and recycled CO_2 (refs 9, 19–21). Compound-specific isotope analyses by GC-IRMS will allow such factors to be distinguished⁵ from other effects such as the transport of 'old' photosynthate from other parts of the plant²². Despite the large inter-species variation of up to 8‰, the differences in $\delta^{13}\text{C}$ between *n*-alkane homologues from a particular tree species were small (generally within 2σ), except for *Acer pseudoplatanus*, which showed a range of 5.2‰; this leaf sample, however, was extensively coated with mould, which could have contributed *n*-alkanes with a different isotope fractionation.

We can draw more specific conclusions about the main inputs to sedimentary carbon from the differences in $\delta^{13}\text{C}$ values of the different tree species than was previously possible using a simple biomarker approach. Figure 2 shows the *n*-alkane distributions of two of the leaf waxes analysed and of two Ellesmere sediment samples. The leaf-wax *n*-alkane distributions are both similar to those of the two sediment samples, especially in the carbon-number range C_{25} to C_{29} . The mean $\delta^{13}\text{C}$ for the *Aesculus* *n*-alkanes, however, was −38.4‰, much more negative than any of the sedimentary *n*-alkanes (Table 1), whereas the mean $\delta^{13}\text{C}$ for *Salix* was −35.0‰, much closer to the values observed in the sediments. Therefore *Aesculus* can be discounted as the main source of the sedimentary *n*-alkanes, and hence of the sedimentary carbon of the lake, whereas *Salix* could represent a small but significant input. These conclusions rest on our data being typical for these species and require that the alkanes surviving mineralization and diagenesis did not fractionate extensively⁴, and that there are no other important inputs of *n*-alkanes to the sediments.

Compound-specific carbon isotope analyses by GC-IRMS should provide new insights into the isotope fractionation processes that affect $\delta^{13}\text{C}$ values of plant organic matter, which will, in turn, clarify understanding of carbon fluxes within bio-

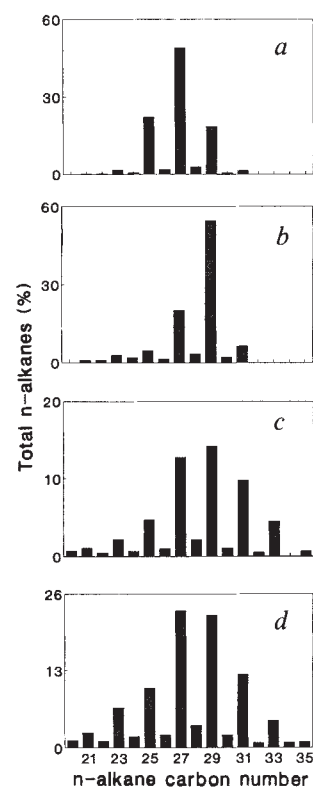


FIG. 2 Total *n*-alkanes (%) as a function of *n*-alkane carbon number for two leaf-wax extracts and two Ellesmere sediment extracts from differing depths. a, *Salix alba*; b, *Aesculus hippocastanum*; c, Ellesmere sediment, 0–5 cm depth; d, Ellesmere sediment, 10–15 cm depth.

geochemical systems generally. This approach will be more discriminatory than conventional combustion-IRMS of bulk organic matter or conventional biomarker analyses alone. For example, investigations of the fate of leaf litter in plant-soil systems would benefit from the analysis of individual components such as lignin monomers. The molecular approach to carbon cycling on land should now be pursued using combined biomarker and stable isotope techniques. □

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Identification of the skeletal remains of a murder victim by DNA analysis

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THERE is considerable anthropological and forensic interest in the possibility of DNA typing skeletal remains. Trace amounts of DNA can be recovered even from 5,500-year-old bones and multi-copy human mitochondrial DNA sequences can frequently be amplified from such DNA using the polymerase chain reaction (PCR)^{1,2}. But given the sensitivity of PCR, it is very difficult to exclude contaminating material. We now report the successful identification of the 8-year-old skeletal remains of a murder victim, by comparative typing of nuclear microsatellite markers³⁻⁵ in the remains and in the presumptive parents of the victim. This analysis establishes the authenticity of the bone DNA and the feasibility of bone DNA typing in forensic investigations.

A 15-year-old caucasian female was murdered in 1981 and her body wrapped in a carpet and buried. The skeletal remains were discovered in 1989. The identity of the victim was provisionally established by a facial reconstruction from the skull and from dental records. To confirm the identification, DNA analysis was attempted. To minimize the risk of contamination, a 5-g fragment of femur was sand-blasted to remove the surface 1-2 mm of bone and divided in two. Each sample was pulverized and DNA extracted as described previously^{1,2}. To determine the quantity and quality of human DNA, aliquots of each sample were electrophoresed alongside known amounts of human genomic DNA, either before or after denaturation. Total DNA was visualized by staining with ethidium bromide and human DNA detected by Southern blot hybridization with a human Alu repeat probe⁶ (Fig. 1). About 5 µg DNA were recovered in each extract, but only a small proportion (roughly 10%) was of human origin, the remainder presumably deriving from bacterial and fungal contamination of the remains. The human DNA component was severely degraded, with almost all single-stranded DNA fragments smaller than 300 nucleotides.

Therefore, DNA typing by Southern blot hybridization with minisatellites⁷, or by amplification of minisatellites by the polymerase chain reaction (PCR)⁸ was not possible. The bone DNA extracts were therefore typed by PCR amplification of polymorphic microsatellite markers, based on (CA)_n repeats, with very short alleles³⁻⁵. Because of the relatively low variability of these markers⁵, six different microsatellite loci were amplified (under containment conditions designed to minimize the risk of contamination⁹) and typed by agarose gel electrophoresis (see Fig. 2, Table 1). All six loci were reproducibly amplified from both bone DNA extracts. The samples were heterozygous at five of the six loci tested.

Microsatellite PCR products from the femur DNA were compared with the corresponding DNA profiles amplified from blood DNA from the presumptive mother (M) and father (F) of the victim, by electrophoresis both on agarose gels and on polyacrylamide DNA sequencing gels (Fig. 2). Both gel systems gave concordant results. Agarose gels gave simpler profiles compared with the multiband pattern per allele on sequencing gels, which probably arise by polymerase slippage at CA repeats during amplification³ and nontemplated nucleotide addition catalysed by *Taq* polymerase¹⁰. All six microsatellite loci gave results fully consistent with the femur DNA being derived from an offspring of M and F, with no instances of a femur microsatellite allele which could not be attributed to M and/or F.

The statistical weight of the evidence linking the skeletal remains to the presumptive parents M and F was evaluated using published caucasian microsatellite allele frequencies (Table 1). The assumption of Hardy-Weinberg equilibrium at

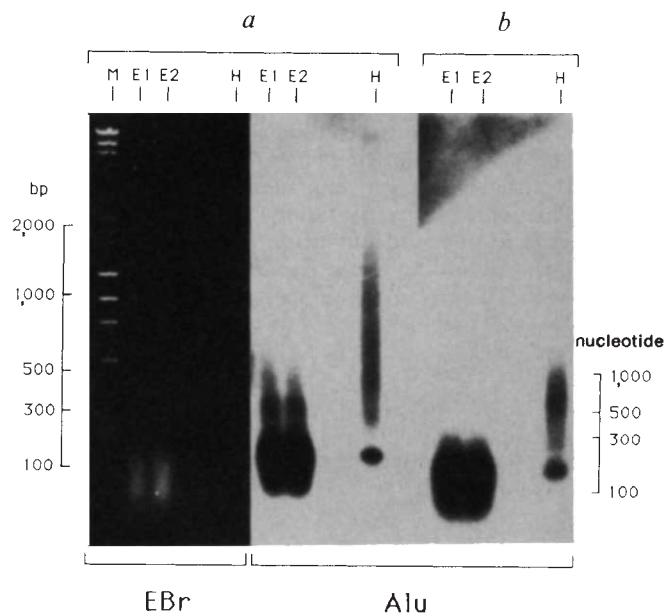


FIG. 1 Assessment of the quantity and quality of human DNA recovered in the femur DNA extracts from the victim. 1/5 of each extract (E1, E2) was electrophoresed alongside 20 ng human genomic DNA (H) digested with *Sau3A*1. DNA electrophoresed either in the native state (a) or denatured (b) was visualized by staining with ethidium bromide (EBr) and human DNA detected by Southern blot hybridization to an Alu probe (Alu). M, marker DNA (λ DNA $\times$ *Hind*III plus Φ X174 DNA $\times$ *Hae*III).

METHODS. DNA in b was denatured with 0.15M NaOH, 10 mM EDTA before electrophoresis through a 1.5% agarose gel. After electrophoresis, DNA was denatured, transferred to Hybond N (Amersham) and hybridized to a ³²P-labelled consensus human Alu probe prepared by denaturation, renaturation to low C₀t and S₁ nuclease digestion of human genomic DNA⁶; this probe was used instead of a cloned human Alu probe to eliminate the risk of contaminating *Escherichia coli* host DNA which might cross-hybridize to bacterial DNA in the femur extracts. Hybridization conditions are described elsewhere⁷.

TABLE 1 Statistical evaluation of the microsatellite evidence linking the skeletal remains to the presumptive parents

Micro satellite	Chromosome	Allele sizes in femur, nt†	Total number individuals in database	Femur allele frequency	Upper 95% limit of allele frequency	Likelihood ratio offspring: unrelated‡	Likelihood ratio (95% frequency limits)‡	Micro satellite reference
Actin	15	72	37	0.11	0.18	21	9	3
TG10	18	100	29	0.16	0.25	2	1	*
		98		0.43	0.54			
Mfd3	1	141	41	0.35	0.45	5	3	4
		131		0.14	0.22			
Mfd5	19	153	75	0.15	0.21	100	34	4
		149		0.03	0.07			
Mfd49	15	96	60	0.02	0.05	58	13	12
		88		0.13	0.19			
Mfd64	1	102	59	0.01	0.04	184	24	13
		88		0.08	0.13			
					Cumulative:	2×10^8	2×10^5	

* I.C.G. and A.J.J., unpublished data. The amplicon sequences for TG10 are CCACAGGTAGAGCCCCTTGG and TAAAGGCACCAGATCCTTCG. The TG10 locus shows 5 alleles with lengths (frequencies in 29 British caucasians) of 108 (0.02), 102 (0.21), 100(0.16), 98(0.43) and 94 base pairs (bp) (0.19).

† Allele lengths were determined from DNA sequencing gels (see Fig. 2) and verified by repeating PCR amplifications with the other amplicon labelled with ^{32}P , followed by sequencing to estimate allele lengths of both the CA and GT strands of the amplified microsatellite. Allele length estimates derived from agarose gel electrophoresis of PCR products using $\Phi\text{X174 DNA} \times \text{HaeIII}$ as marker were within 2 bp of those determined from sequencing gels (data not shown).

‡ Likelihood ratio of the probabilities of obtaining the precise combination of alleles seen in the presumptive parents M and F and in the skeletal DNA sample, under the alternative assumptions that the skeletal DNA is either derived, or not derived, from an offspring of M and F. If both parents are heterozygous, this is given by $1/8q_1q_2$, where q_1 and q_2 are the population frequencies of the two alleles in heterozygous bone DNA. If one or both parents are homozygous, this likelihood ratio becomes $1/4q_1q_2$ or $1/2q_1q_2$, respectively. If the bone DNA is homozygous for an allele of frequency q , the three likelihood ratios are $1/4q^2$, $1/2q^2$ and $1/q^2$, respectively.

each locus and statistical independence of different loci appear to hold true for microsatellite and simple tandem repeat loci investigated to date¹¹. For each locus, the likelihood ratio was determined for the probability of obtaining the M, F and bone genotypes if the skeletal remains were those of an offspring of M and F versus the corresponding probability if the bone genotype was not related to M and F. The latter possibility would arise either if the skeletal remains were not those of an offspring of M and F, or if the bone DNA typing information was derived from human contamination introduced when handling the bones or subsequently during DNA analysis. This likelihood ratio in favour of identification varies substantially from locus to locus according to the population frequencies of the femur alleles (Table 1). The cumulative likelihood ratio for all

six loci is very high (2×10^8). But the population databases for allele frequencies at these loci are currently relatively small and to compensate for sampling errors in allele frequency estimates, the likelihood ratios were re-evaluated after adjusting all allele frequencies to their upper 95% confidence limits (Table 1). The cumulative likelihood ratio remains high (2×10^5), although it is possible that this estimate could be perturbed if there are major allele frequency differences between the caucasian populations represented in the published databases and in the local population relevant to this case (South Wales). Nevertheless, the DNA data establish with a high degree of confidence that the murder victim was indeed the daughter of M and F.

This analysis is the first example of the successful identification of skeletal remains by bone DNA analysis and provides

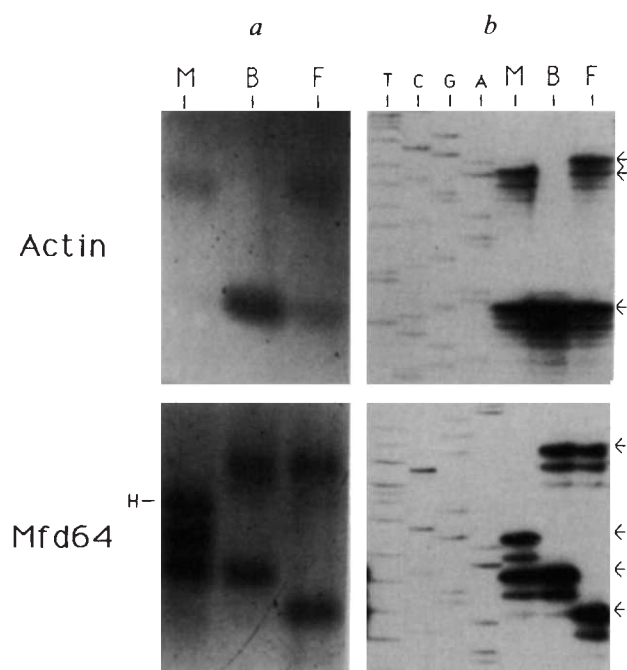


FIG. 2 Examples of microsatellite typing in the bone DNA (B) compared with the presumptive mother (M) and father (F) of the victim. Alleles at two loci (actin, Mfd64) were amplified by PCR and typed either native on an agarose gel (a) or denatured on a DNA sequencing gel (b). For both loci, the genotype of the femur was compatible with the DNA being from an offspring of M and F. H, heteroduplex of the two maternal alleles arising through over-amplification of the Mfd64 locus. Allele lengths in (b) were determined from the major PCR product of each allele (arrowed).

METHODS. For each locus, an aliquot of femur DNA extract containing approximately 10 ng human DNA was amplified in a 30 μl reaction for 33 cycles as described previously⁹. Larger amounts of femur DNA consistently inhibited amplification (data not shown) and were avoided. Parental blood DNA samples (6 ng) were likewise amplified. Samples (25 μl) of each PCR reaction were electrophoresed through a 3% NuSieve agarose, 1.2% Sigma type I agarose gel in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH8.3) and PCR products visualized by staining with ethidium bromide. Aliquots (1 μl) of each PCR reaction were reamplified for four cycles in a 10 μl PCR reaction, with one of the amplicons ^{32}P -labelled using T4 polynucleotide kinase and [γ - ^{32}P] ATP. Labelled products were denatured, electrophoresed through a DNA sequencing gel alongside an M13mp18 sequencing ladder (T, C, G, A) and visualized by autoradiography. Control amplifications lacking DNA consistently gave negative results (not shown). The heteroduplex band H was verified by showing that this band only arose at high PCR cycle number and that it could be generated by denaturation/renaturation of amplified alleles (data not shown).

very strong evidence that the human DNA extracted from the bone was from the victim and did not arise through contamination. Although bone DNA typing is likely to have a major impact on forensic and anthropological investigations, we would however introduce two notes of caution. First, it is unclear what proportion of skeletal remains will yield sufficient human DNA to enable nuclear markers such as microsatellites to be typed. Second, in the case of an apparent exclusion based on bone DNA analysis, it may be impossible to determine whether the exclusion is authentic or is inadvertently derived from contaminating material. □

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Position-dependent expression of two related homeobox genes in developing vertebrate limbs

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MANY genes that control pattern formation in insects contain a conserved homeobox region which encodes a domain involved in DNA binding¹. One approach to understanding pattern formation in vertebrates is to examine the role of homeobox-containing genes in the developing limb². Two such genes, *Hox-7.1* and *Hox-8.1*, are expressed in distal mesoderm, but not in the proximal core, of mouse forelimb (refs 3, 4, and D.R.D., manuscript in preparation). The proximodistal cartilage pattern in the chick wing is progressively determined in the distal mesoderm, which is maintained as a 'progress zone' by the overlying apical ectodermal ridge⁵. Indeed, proximal cells are reprogrammed to form distal structures when placed in the progress zone⁶ and we therefore expect that genes involved in controlling limb pattern should be activated in such grafts. We tested this requirement for *Hox-7.1* and *Hox-8.1* in mouse limb mesoderm placed in chick wing buds. Our results reported here indicate that both genes are rapidly activated by a signal from the apical ectoderm. These properties, taken with the DNA-binding properties of the homeodomain, strongly suggest that *Hox-7.1* and *Hox-8.1* have fundamental roles in limb-pattern formation.

At the earliest stage when the forelimbs are visible (9.5 days post coitum), *Hox-7.1* is expressed throughout the bud, whereas *Hox-8.1* expression is confined to the ventral and distal ectoderm and the ventral mesoderm. By the stage of limb-bud outgrowth at which the experiments were done (Fig. 1), the patterns of gene expression have changed. *Hox-8.1* transcripts, but not those

of *Hox-7.1*, are abundant in the apical ridge. Both genes are expressed in the distal mesoderm where, on the basis of *in situ* hybridization signal, *Hox-7.1* transcripts seem to be more abundant than those of *Hox-8.1*. Transcripts of both genes have a gradient of abundance, highest distally, and this pattern persists for at least another 2 days. Neither gene is detectably expressed in the proximal core mesoderm. But both are expressed in a well-defined anterior proximal mesodermal region. Forelimb and hindlimb buds show the same patterns of expression, but the forelimb is about 24 h more advanced.

When fragments of mouse mesoderm were placed beneath the apical ectodermal ridge at the tip of early wing buds (Fig. 2), the grafted cells remained near the tip of the chick limb bud (see also ref. 7). Cells in fragments taken from the tip of mouse limb buds continued to express *Hox-7.1* (six out of six grafts) and most expressed *Hox-8.1* (five out of six) 24–26 h after grafting (Fig. 3). Transcripts for the two genes were now also present in tissue taken from proximal regions of mouse limb buds where the genes were not expressed before grafting (Fig. 3; four out of four grafts). Even though the mouse tissue was placed under the apical ridge in random orientation, all grafts, whether of distal or proximal origin, showed a similar pattern of gene expression. In each case, *Hox-7.1* transcripts were much more abundant than those of *Hox-8.1* and both genes showed graded expression greatest in the part of the graft closest to the apical ectoderm. Indeed, several large grafts contained transcripts of both genes distally but no expression in the proximal part of the graft. *Hox-8.1* transcripts were confined to a rim of grafted cells just below the apical ridge (Fig. 3k, l). In the graft

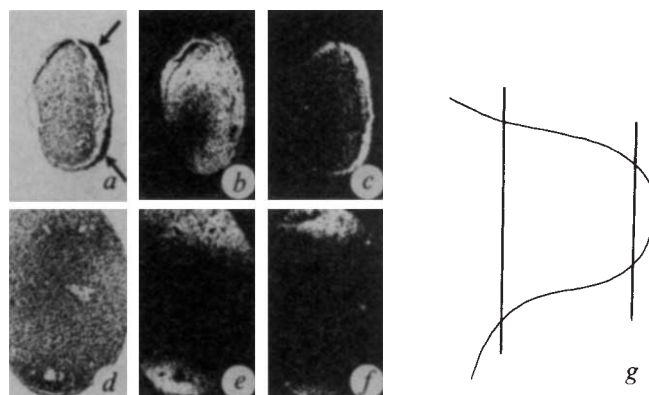


FIG. 1 The expression of *Hox-7.1* and *Hox-8.1* messenger RNA in a mouse limb bud at a stage from which tissue was taken for grafting. *a-f*, Bright-field and dark-field photographs of sections through the distal part (*a-c*) and proximal part (*d-f*) of a hindlimb bud from an 11.5-day-old embryo. Grafts were taken from this stage or from the equivalent stage (10 days) of forelimb development. Anterior, up; ventral, right. The apical ectodermal ridge is indicated by an arrow. Sections were hybridized *in situ*, using methods described previously¹⁶, with specific probes complementary to the 3' ends and untranslated regions of the mouse *Hox-7.1* and *Hox-8.1* genes (ref. 3, and A. P. Monaghan *et al.*, manuscript submitted). The dark-field photographs show the patterns of expression of *Hox-7.1* (*b, e*) and *Hox-8.1* (*c, f*). Magnification $\times 50$. *g*, Diagram showing planes of sections represented in photographs. Anterior is up, ventral below plane of page. As a result of ventral curvature of the bud, the distal sections pass through material closer to the distal tip of the bud on the ventral (right) side compared with the dorsal side (left of the section). Note that *Hox-7.1* is abundantly expressed throughout the distal material used as grafts, whereas at this stage of development *Hox-8.1* is more weakly expressed and principally in the part of the distal material immediately below the apical ridge. Note also that neither dorsal nor core material from proximal regions used as grafts expressed *Hox-7.1* or *Hox-8.1*. *In situ* hybridization using probes to *Hox-7.1* and *Hox-8.1* confirmed that cells in fragments dissected from the tip expressed transcripts of both homeobox genes, whereas cells in fragments dissected from the proximal dorsal regions of mouse limb buds expressed these genes weakly, if at all, or only a few cells expressed the genes (data not shown).

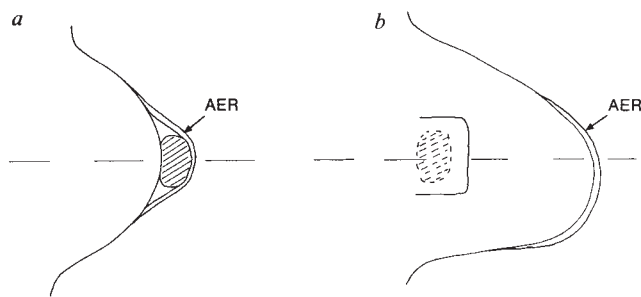


FIG. 2 Diagram showing mouse limb bud mesenchyme (hatched) grafted to chick wing buds which provides an assay system because the signalling mechanisms of bird and mammalian limb buds are compatible^{17,18}. *a*, Mouse tissue placed beneath the apical ectodermal ridge (AER) at the tip of stage 20 wing bud. *b*, Mouse tissue placed proximally beneath a flap cut into the dorsal surface of stage 22 wing bud. Horizontal broken line indicates the plane of sections in Fig. 3.

METHODS. Limb buds from mouse embryos (C57Bl/6) were placed in 2% trypsin at 4 °C for 1 h, then transferred to minimal essential medium +10% fetal calf serum and the ectoderm removed. Fragments of mesenchyme were then dissected and grafted to chick wing buds. Fertilized chicken eggs (Ross White) were incubated at 38 °C and staged according to Hamburger and Hamilton¹⁹.

in which *Hox-8.1* expression was not detected, no mouse cells lay close to the apical ridge. These patterns of expression faithfully reproduced those found in normal mouse limb buds except that the intensity and extent of *Hox-8.1* expression was less in the grafts than in intact mouse limb buds.

To assess whether the activity of these genes is a direct or indirect response to position, we investigated their expression much earlier after grafting. Proximal mesoderm taken from

11-day-old mouse forelimbs was placed at the tip of chick wing buds and the distribution of transcripts was compared in grafts after 5 h and 24 h. Five hours after the operation, the protruding graft had not completely healed into the bud, although there was close apposition between the grafted cells and the apical ectodermal ridge (Fig. 3). Nevertheless, the proximal mouse tissue now placed at the tip already contained detectable levels of transcripts of *Hox-7.1* and *Hox-8.1* immediately below the distal ectoderm (Fig. 3*a-d*). By 24 h, the transcripts of both genes were much more abundant over a wider region but still with maximal expression distally. The rapidity with which transcripts of both genes became detectable in these grafts is consistent with the view that their activation is a direct consequence of the new position of the cells.

In reciprocal experiments, fragments of mouse mesoderm were placed in proximal regions of slightly older wing buds (Fig. 2). Transcripts of *Hox-7.1* and *Hox-8.1* were not detectable in any proximal grafts (Fig. 3*m-t*; *n* = 9) except for one proximal to proximal graft which contained a very low level of *Hox-7.1* transcripts (Fig. 3*q, r*). No activity was observed in two grafts that came to lie close to the ventral surface of the limb (see Fig. 3*s, t*), indicating that the activity observed in distal grafts is not a general reaction to the proximity of surface ectoderm. These results indicate that even tissue that is actively expressing *Hox-7.1* and *Hox-8.1* at the time of grafting and that would continue to do so if placed distally, becomes inactive in response to its position in the proximal part of the wing.

Our results indicate that *Hox-7.1* and *Hox-8.1* are expressed in response to graded proximodistal positional cues in the distal region of the limb. The rapid appearance of the transcripts immediately below the apical ridge and the subsequent spread of the regions of expression deeper into distal mesenchyme, resulting in a gradient in abundance of transcripts 24 h after grafting, suggests that the genes are activated in response to a

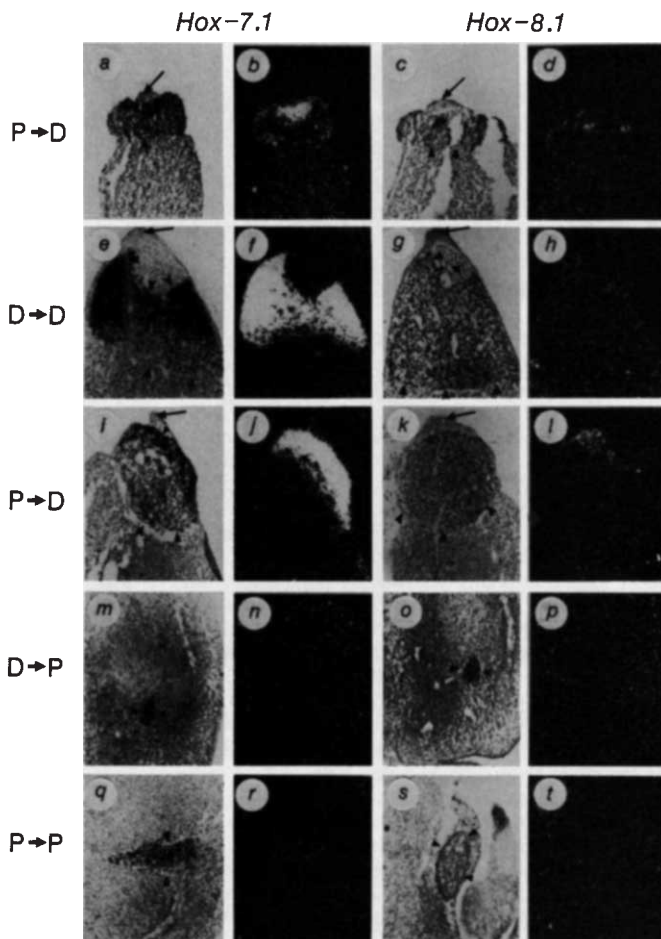


FIG. 3 The expression of *Hox-7.1* and *Hox-8.1* mRNA in mouse limb bud tissue grafted proximally or distally into chick wing buds. The figure shows bright-field and corresponding dark-field photographs of sections cut along the long axis of developing chick limbs in the regions containing the grafts. The relevant sections were selected by reference to an alternate series of sections that were stained with Biebrich scarlet to show the location of the mouse cells⁷. The graft is generally stained slightly darker than the host tissue and the limits are indicated by arrow-heads where appropriate. The apical ridge is arrowed. The cells placed under the apical ridge remained near the tip of the chick limb bud and 6 days later were found in ectopic cartilage and in soft tissues in distal regions of the wing adjacent to chick digits that had developed (see also ref. 7). The cells placed proximally were found towards the centre of the base of the wing at 24 h. In most cases the graft was surrounded on all sides by chick mesoderm which had begun to differentiate into muscle and cartilage of the upper wing. Compared with grafts placed at the tip, grafts placed proximally were much less extensive suggesting that growth was reduced (see also ref. 20). Sections on the left were hybridized with a probe for *Hox-7.1* and nearby sections, shown on the right, with a probe for *Hox-8.1*. Limbs shown on the top row, *a-d*, were fixed 5 h after grafting. All other limbs were fixed 24–26 h after grafting. The various grafts are indicated in the left margin (mouse-donor site → chick-host site: P, proximal; D, distal). Proximal-distal grafts, *a-d, i-l*; distal-distal grafts, *e-h*; distal-proximal grafts, *m-p*; proximal-proximal grafts, *q-t*. Note that in *g* and *h*, most of the graft does not lie adjacent to the apical ridge and *Hox-8.1* is barely expressed above background, whereas *Hox-7.1* is strongly expressed in the nearby sections *e, f*. When the graft was directly below the apical ridge as in *k* and *l*, *Hox-8.1* was expressed in cells immediately next to the ridge. All photographs magnified ×80. All the sections shown in this plate are roughly comparable as they were hybridized in the same experiment, given that the same autoradiographic exposure and all the dark-field views were photographed and printed under the same conditions. Sections hybridized with a control sense probe to *Hox-8.1* gave, in both mouse and chick tissue, only an extremely low signal, which was taken as the background level.

signal emanating from the apical ectoderm. As *in situ* hybridization does not detect very low amounts of transcripts, it is unclear whether the activation of these genes represents stimulation or *de novo* expression. The characteristic amounts of activity and regions of expression of *Hox-7.1* and *Hox-8.1* may be specified by different threshold responses to the signal. One candidate is BMP-2A, a member of the transforming growth-factor family, which is expressed in the ventral ectoderm of the early mouse limb and later confined to the apical ridge⁸.

The rapid activation of *Hox-7.1* and *Hox-8.1* in tissue placed in the progress zone is consistent with a primary function for these genes in limb-pattern formation. Many other genes are expressed in the limb bud that may act with, or be regulated by, *Hox-7.1* and *Hox-8.1*. These include several homeobox-containing genes⁹ and others that are likely to have morphogenetic functions, retinoic acid receptors and binding protein^{10,11}, int-related¹² and AP-2 (ref. 13). In contrast to *Hox-7.1* and *Hox-8.1*, at least some of these genes seem not to be rapidly

responsive to positional cues. The activity of a chick gene homologous to the *XlHbox1* gene of *Xenopus* appears to be cell autonomous in reciprocal anteroposterior grafts in limbs¹⁴. In addition, new transcripts of chick genes homologous to genes of the mouse HOX-4 complex, which are induced in distal mesoderm of chick wing buds that will give a duplicated pattern, only reach detectable levels after 16–24 h suggesting an indirect response to positional signals¹⁵.

To test critically the hypothesis that *Hox-7.1* and *Hox-8.1* play a part in proximodistal pattern formation in the limb, it will be necessary to examine the effects of changes in gene expression on limb development. The ability to regulate *Hox-7.1* and *Hox-8.1* in tissue grafted to the limb provides an assay which may be useful in identifying the signals and responsive elements that control their developmental expression. Ultimately manipulation of these controls may provide a means to examine the roles of *Hox-7.1* and *Hox-8.1* in the development of the limb skeleton. □

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Sodium channel density in hypomyelinated brain increased by myelin basic protein gene deletion

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TROPHIC control over the expression and membrane distribution of voltage-dependent ion channels is one of the principal organizing events underlying the maturation of excitable cells. The myelin sheath is a major structural determinant of regional ion channel topography in central axons^{1,2}, but the exact molecular signals that mediate local interactions between the oligodendrocyte and axolemma are not known. We have found that large caliber fibre pathways in the brain of the mutant mouse *shiverer* (*shi*, gene on chromosome 18), whose developmental fate of myelination is averted by deletion of five exons in the myelin basic protein gene^{3–5}, have a striking excess of sodium channels. As cytoplasmic membranes of *shiverer* oligodendroglia still adhere to axons^{6–8}, the evidence indicates that myelin basic protein or a myelin basic protein-dependent glial transmembrane signal associated with compact myelin formation, rather than a simple glial–axon contact inhibition or an intrinsic genetic program of neuronal differentiation, could be critical in downregulating sodium channel density in axons. Here we use the *shiverer* mutant to show that mature central nervous system projection neurons with large caliber unmyelinated fibres sustain functional excitability by increasing sodium channel density. This axon plasticity, triggered by the absence of a single glial protein, contributes to the unexpectedly mild degree of neurological impairment in the mutant brain without

myelin, and may be a potentially inducible mechanism determining the recovery of function from dysmyelinating disease.

We prepared autoradiograms of adult *shi/shi* and *+/+* brain sections and performed regional binding assays with tritiated saxitoxin, which binds externally to the membrane-bound⁹ but not to the free intracellular form¹⁰ of the sodium channel α subunit, the peptide requisite for ion conduction¹¹. Autoradiography revealed striking increases (2.2–4.7-fold) in silver grain density, reflecting specific ³H-saxitoxin binding over all hypomyelinated *shiverer* fibre tracts when compared with *+/+* brains (Fig. 1). Within mixed grey matter regions the increases are less striking (1.1–1.4-fold) and reflect the proportional content of hypomyelinated fibres of passage (Table 1). In the most homogeneous nuclear regions, where only a small fractional area consists of penetrating hypomyelinated fibres, there is little difference between genotypes. One such region, the outer molecular layer of cerebellar cortex, comprises an essentially pure population of unmyelinated parallel fibres showing one of the highest sodium channel densities in the brain; binding in this layer is unaltered by the mutant gene, indicating that the channel density increase is nonuniform and predominates in hypomyelinated axon pathways.

To determine whether the density increase in the mutant was due to an alteration in the number of receptors, their affinity, or both, we performed specific binding experiments on freshly dissected central nervous system (CNS) homogenates solubilized in excess scintillant in which lipid quenching is essentially zero. Samples composed of hypomyelinated fibres (optic nerve), mixed cellular neuropil (neocortex) and whole brain were used for saturation studies. Specific binding was saturable in both genotypes. Scatchard analysis showed the presence of a single binding site, and toxin affinity was not significantly different between genotypes (Fig. 2). In contrast, the number of binding sites ($B_{\max}$) in mutants was 3–4-fold higher in optic nerve and 1.6-fold higher in neocortex than it was in control mice. The level of specific toxin binding in heterogeneous brain areas was 10–35% higher in the mutant than in corresponding control regions (Table 2).

The ^3H -saxitoxin density increase in hypomyelinated pathways may not be confined to axons, and we do not exclude the possible addition of sodium channels at adjacent non-neuronal sites. Oligodendroglia do not express sodium channels¹², but astroglia show ^3H -saxitoxin binding and voltage-dependent sodium currents¹³ and are relatively increased in *shiverer* pathways^{3,6,8}. Although astrocytes may contribute a fraction of the ^3H -saxitoxin binding increase in hypomyelinated brain, two lines of evidence favour an axonal origin. First, instead of flaccid

or spastic paralysis, the *shiverer* mutant shows an unexpected neurological phenotype of normal motor strength and tone, consistent with intact impulse conduction in corticospinal pathways. Astroglial sodium channels cannot account for persistent conduction across inexcitable internodal membrane by any known mechanism. Second, an increase in axolemmal sodium channels would predict associated elevations in axonal ($\text{Na}^+ + \text{K}^+$)ATPase and cytochrome oxidase activity to fuel the metabolic demand of restoring ionic gradients; both have been identified in *shiverer* pathways¹⁴ (J.L.N., unpublished results). We conclude that a substantial fraction of the ^3H -saxitoxin density increase is localized on central axons.

Although intermediate stages of axolemmal sodium channel arrangement during myelinogenesis have not been fully clarified in mammalian CNS axons, glia are important determinants of the final topography. Ultrastructural observations of external face intramembraneous particle distributions indicate that where myelin does not form, the axolemma fails to differentiate, and that where myelin forms abnormally, there are corresponding abnormalities in the axolemma^{1,2,15}. As sodium ion-mediated impulse conduction precedes myelination in developing CNS pathways^{16,17}, and few if any sodium channels are found along the acutely desheathed adult internode¹⁸, acquisition of myelin must be accompanied by a controlled reduction and rearrangement of the native distribution of axolemmal sodium channels in the recipient neuron. There are two major hypotheses on the cellular origin of signals regulating this rearrangement. The neuronal hypothesis, based on indirect evidence that intramembraneous particle clusters form in the absence of myelinating cells¹⁵, specifies that some degree of specialized nodal-internodal architecture arises from constitutive gene expression in the differentiating neuron. The glial hypothesis, supported in part by inhibitory responses of axons to specific oligodendroglial molecules¹⁹, stipulates a secondary suppression of internodal channels after contact with the myelinating cell. If sodium channel arrangement in axons were regulated solely by the parent neuron according to the first hypothesis, ^3H -saxitoxin binding in myelin-deprived fibres would not differ from control fibres. This prediction is contradicted by our results, which favour a mechanism of axon channel remodelling directed by glia, wherein the developing neuron maintains an ion channel density optimal for its caliber²⁰ until an inhibitory glial signal is received.

TABLE 1 Gene-linked alterations in specific ^3H -saxitoxin binding to *shiverer* and $+/+$ brain regions (fmol mg^{-1} protein)

Region	$+/+$	<i>shi/shi</i>	Per cent of control
Myelinated tracts:			
Optic nerve	5.8 $\pm$ 6.6	27.2 $\pm$ 11.1	468.9*
Corpus callosum	77.7 $\pm$ 63.6	178.6 $\pm$ 55.4	229.9*
Anterior commissure	58.8 $\pm$ 55.8	176.2 $\pm$ 21	299.6*
Fimbria	54.6 $\pm$ 28.6	161.7 $\pm$ 40.9	296.2*
Alveus	72.1 $\pm$ 34.9	233.8 $\pm$ 59.2	324.3*
Internal capsule	67.2 $\pm$ 31	43.8 $\pm$ 34.1	214.0*
Fornix	52.1 $\pm$ 34.5	114.4 $\pm$ 39.3	219.5†
Unmyelinated:			
Cerebellar molecular layer	340.8 $\pm$ 37.3	351.5 $\pm$ 43.5	103.1 (NS)
Predominantly nuclear:			
Neocortex	138.1 $\pm$ 22.5	193.9 $\pm$ 31.5	140.4*
Substantia Nigra	196.5 $\pm$ 41.7	248.4 $\pm$ 37.8	126.4*
Periaqueductal grey	132.6 $\pm$ 33	184.7 $\pm$ 32	142.9*
Cerebellar granule cell	89.3 $\pm$ 24.2	121.8 $\pm$ 38.4	136.4*
Thalamus	118.3 $\pm$ 47.1	151.1 $\pm$ 44.9	127.7†
Hippocampus	139.8 $\pm$ 41.7	155.7 $\pm$ 36.2	111.4 (NS)
Septum	141.0 $\pm$ 62.2	159.4 $\pm$ 61.5	113.1 (NS)
Hypothalamus	150.8 $\pm$ 64	170.4 $\pm$ 42.4	119.0 (NS)
Sup. Colliculus	175.6 $\pm$ 51	232.0 $\pm$ 43.8	134.0 (NS)
Mixed fibre/nuclear			
Anterior midbrain	58.7 $\pm$ 24.3	161.0 $\pm$ 27	274.3*
Pons	67.6 $\pm$ 31.7	162.5 $\pm$ 38.5	282.1*
Cerebellum	162.7 $\pm$ 23	197.8 $\pm$ 30.8	121.6*
Striatum	96.6 $\pm$ 48	137.9 $\pm$ 48	142.7*
Midbrain	83.7 $\pm$ 24	169.7 $\pm$ 27.1	202.8*
Whole brain sections	129.2 $\pm$ 39.7	175.2 $\pm$ 37.5	135.6*

Integrated optical densitometry was used to quantitate silver grain densities. The analog video signal was corrected for background illumination and digitized (Loates) so that the optical density of each 3×3 micron area of the film, corresponding to one pixel, represented a value from 0–225. The absolute value for tissue densities of ^3H -saxitoxin was determined using a calibration curve derived from standards exposed on the same film. Specific binding was determined by subtracting nonspecific from total binding using optical overlays. Densities were sampled from 30 brain regions at 8 ascending levels of the neuraxis in 3 mice of each genotype by taking the mean of triplicate square 'punches' within the perimeter. Boundaries of heterogeneous regions were circumscribed and measured as a unit. Specific binding in each region was averaged across all samples and compared by genotype using the Student's two-tailed *t*-test. Quantitative autoradiography with tritiated ligands is corrected for differential β emission absorption by lipids because tritium efficiency is lower over myelinated regions²⁸. Although *shiverer* oligodendrocytes are not numerically reduced³, the secondary wrappings only loosely envelop axons, and total lipid levels are ~20–30% lower in the mutant's CNS (ref. 29). When the calculated densities are corrected using a maximal quench correction factor of 0.3, specific ^3H -saxitoxin binding within hypomyelinated *shiverer* fibre tracts remains significantly high compared with $+/+$ pathways. Two geometric factors, a potential reduction in either fibre diameter or interaxonal spacing within the pathway, do not apparently account for the density increase. Mean axon circumferences were reduced by <6% in a sample of 1,800 *shi/shi* (2.25 μm) and $+/+$ (2.39 μm) optic nerve axons (H. D. Shine, personal communication), and white matter tract widths in Luxol Fast blue stained sections were directly superimposable, suggesting that fibre diameter and packing density are little affected by the loose glial wrapping.

* $P < 0.001$; † $P < 0.05$; NS, not significant.

TABLE 2 Specific binding of ^3H -saxitoxin to $+/+$ and *shi/shi* brain regions *in vitro*

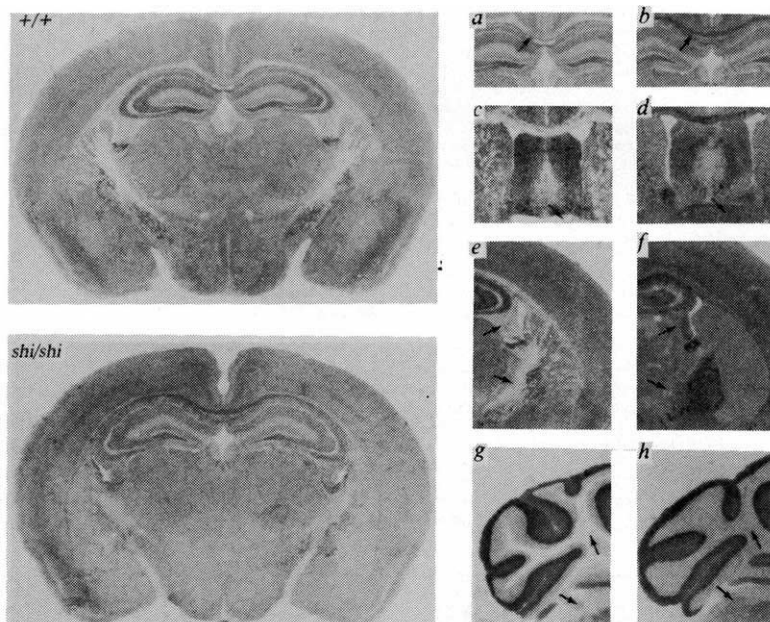
Tissue	Specific binding (fmol mg^{-1}) $+/+$	<i>shi/shi</i>	Per cent of control
Neocortex ($n=5$)	285.9 $\pm$ 12.9	385.5 $\pm$ 36.8	135*
Hippocampus ($n=3$)	464.3 $\pm$ 5.1	512.73 $\pm$ 3	110*
Cerebellum ($n=5$)	466.9 $\pm$ 21.4	547.0 $\pm$ 7.9	117†
Whole brain ($n=4$)	369.8 $\pm$ 19.7	469.7 $\pm$ 32.3	127‡

Means $\pm$ s.e. of the number of experiments in parentheses (each in triplicate determinations). Dissected brain regions were homogenized in incubation buffer (20 mM Tris-HCl, pH 7.4, containing 140 mM *N*-methyl glucamine, 5.4 mM KCl, 2.8 mM CaCl_2 and 1.3 mM MgSO_4) at 4 °C. In preliminary experiments toxin binding at 4 °C was complete at 45 min using constant radioligand concentrations of 5 nM. Specific binding accounted for ~90% of total binding. Specific binding of ^3H -saxitoxin in the presence of increasing concentrations of cellular protein was linear up to 350 μg and freezing did not reduce the linearity or extent of binding. For saturation studies, aliquots of 120–200 μg cellular protein were added to an assay mixture containing 5 nM ^3H -saxitoxin in a final volume of 100 μl . Assays used to define nonspecific binding also contained 1 μM tetrodotoxin. The mixture was incubated for 60 min at 4 °C, terminated by addition of 3.5 ml ice-cold buffer and filtered through GF/C filters. Filters were washed twice with buffer, dried, and the radioactivity assayed by liquid scintillation counting.

* $P < 0.025$; † $P < 0.001$; ‡ $P < 0.05$.

FIG. 1 Left: Autoradiograms of specific ^3H -saxitoxin binding in adult wild-type and *shiverer* mouse brain reveal increases in sodium channel density within hypomyelinated mutant central axon tracts. Right: Regional ^3H -saxitoxin binding in $+/+$ (a, c, e, g) compared with *shi/shi* (b, d, f, h) pathways is indicated by arrows: a–b, corpus callosum; c–d, anterior commissure; e–f, hippocampal fimbria (upper arrow) and internal capsule (lower); g–h, cerebellar white matter (upper) and inferior cerebellar peduncle (lower).

METHODS. ^3H -saxitoxin autoradiography: Coronal cryostat sections (20 microns) were cut from fresh adult (3–4 months postnatal) homozygous male *shi/shi* (C57Bl/6-*shi*) and C57Bl/6 ($+/+$) brains at 8 predesignated levels of the neuraxis rostral to the medulla. Frozen sections were thaw-mounted on gel alumin-subbed slides and dried in a dessicator overnight at 4°C . Slides from each genotype were matched into sets for binding using adjacent cresyl violet-stained sections to verify comparable neuroanatomical planes of section. Matched slides were simultaneously incubated with 5 nM ^3H -saxitoxin (Amersham) at 4°C for 60 min in incubation buffer (20 mM Tris-HCl, pH 7.4, containing 140 mM *N*-methyl glucamine, 5.4 mM KCl, 2.8 mM CaCl_2 and 1.3 mM MgSO_4) according to Mourre *et al.*³⁰. Incubation was terminated by 2×5 -second washes in ice-cold buffer and a quick rinse in distilled water, then sections were dried under cold air. Non-specific binding was determined in adjacent sections by preincubation with $1 \mu\text{M}$ tetrodotoxin (Sigma). Slide pairs of specific and non-specific binding from the two genotypes were then placed side-by-side on the same sheet of Hyperfilm ^3H with glass-mounted ^3H



calibration standards (Microscale, Amersham), exposed in cassettes for 2–3 weeks and developed in the same way.

Shiverer axons present two important clues regarding the nature of this signal and the source of the channels. Although close apposition of glial plasma membranes could present a steric barrier to the free lateral movement of axolemmal cation-selective channel proteins with large external domains (~ 60 nm) in $+/+$ fibres, it is unlikely that a lack of simple physical contact alone could mediate the increase in sodium channel density in the mutant. Despite the absence of multilamellar compact myelin in *shiverer*, the primary oligodendroglial membrane wrapping adheres to the internodal axolemma at irregularly

spaced axoglial junctions with the usual ~ 20 – 30 nm gap; these appositions are even more prevalent in mutant than in wild-type axons⁷. Furthermore, because the absolute number of sodium channels in *shiverer* hypomyelinated pathways is raised, impulse conduction is less likely to be preserved by spatial redistribution of existing nodal channels than by increased stabilization of new channels in the bilayer. Our data therefore suggest that the immediate presence of one or more of the six myelin basic protein isoforms expressed in normal mouse CNS myelin^{21,22} or of other related myelin components whose expression may be linked to that of MBP (for example, proteolipid protein²³ and myelin-associated glycoprotein^{14,24}), but not glial-axonal membrane adhesion alone, is a prime signal regulating sodium channel density in adult axons.

Acute neurological deficits that accompany the demyelinating lesions of multiple sclerosis in human white matter confirm estimates predicting impulse conduction failure at inexcitable regions exposed by the injured oligodendrocyte, but clinical remission without total fibre remyelination²⁵ and signs of ectopic axon hyperexcitability²⁶ (both consistent with increased sodium channel density²⁰) are frequently found in patients recovering from this disease. The evidence for functional axon conduction with increased sodium channel density in the hypomyelinated brain lacking myelin basic protein suggests that demyelinated CNS fibres in multiple sclerosis may regain excitability by a similar mechanism once the inhibitory influence of a myelin membrane protein is removed. The *shiverer* mutation identifies myelin basic protein as a candidate glial suppressor protein involved in the trophic regulation of neuronal sodium channels. Further elucidation of the entire signalling sequence linking glial membrane proteins to the regulation of sodium channel α -subunit gene transcription²⁷ may suggest specific strategies for the clinical management of ion channel gene expression in dysmyelinating disease. □

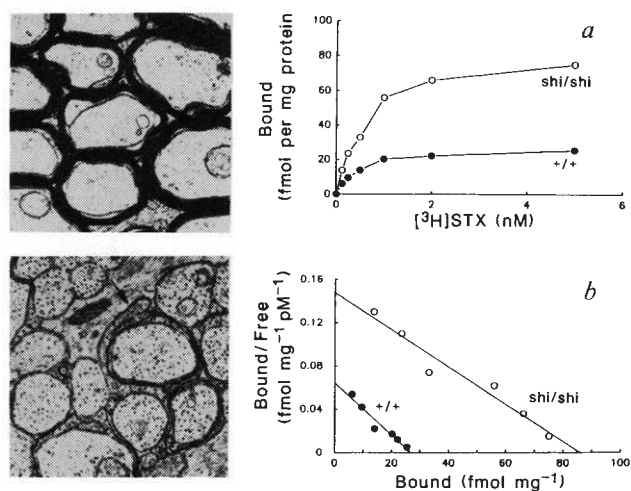


FIG. 2 Left: Ultrastructure of myelinated $+/+$ (upper) and hypomyelinated *shi/shi* fibres without myelin basic protein (lower) in adult optic nerve shows glial adhesion and absence of compact myelin with loose oligodendrocyte wrappings in mutant axons (arrow). Right: a, Representative data from one experiment showing dose-dependent specific binding of ^3H -saxitoxin (STX) to optic nerve axons from $+/+$ and *shi/shi* mice (method described in legend to Table 2). In each experiment optic nerve samples from *shi/shi* ($n=20$) and $+/+$ ($n=12$) mice were pooled to obtain adequate protein specimens for triplicate binding assays. b, Scatchard analysis of data in a gave $K_d=0.42$; $B_{\text{max}}=27.6 \pm 2$ ($+/+$) and $K_d=0.58$; $B_{\text{max}}=85.8 \pm 8$ (*shi/shi*). Repeat assays produced similar values. Identical experiment using neocortex produced values for $K_d=0.29$; $B_{\text{max}}=227.1 \pm 10$ ($+/+$) and $K_d=0.28$; $B_{\text{max}}=376.5 \pm 21$ (*shi/shi*).

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Prevention of HIV-1 IIIB infection in chimpanzees by CD4 immunoadhesin

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THE first step in infection by the human immunodeficiency virus (HIV) is the specific binding of gp120, the envelope glycoprotein of HIV, to its cellular receptor, CD4 (see ref. 1 for review). To inhibit this interaction, soluble CD4 analogues that compete for gp120 binding and block HIV infection *in vitro* have been developed^{2–8}. To determine whether these analogues can protect an uninfected individual from challenge with HIV, we used the chimpanzee model system of cell-free HIV infection. Chimpanzees are readily infected with the IIIB strain of HIV-1, becoming viraemic within about 4–6 weeks of challenge, although they do not develop the profound CD4⁺ T-cell depletion and immunodeficiency characteristic of HIV infection in humans⁹. CD4 immunoadhesin (CD4-IgG), a chimaeric molecule consisting of the N-terminal two immunoglobulin-like regions of CD4 joined to the Fc region of human IgG1 (refs 8, 10), was selected as the CD4 analogue for testing because it has a longer half-life than CD4, contributed by the IgG Fc portion of the molecule. In humans, this difference results in a 25-fold increased concentration of CD4-IgG in the blood compared with recombinant CD4 (ref. 11). Here we report that pretreatment with CD4-IgG can prevent the infection of chimpanzees with HIV-1. The need for a preventative agent is particularly acute in perinatal HIV transmission. As recombinant CD4-IgG, like the parent IgG molecule, efficiently crosses the primate placenta¹⁰, it may be possible to set up an

immune state in a fetus before HIV transfer occurs, thus preventing infection.

Two chimpanzees were pretreated with CD4-IgG (5 mg kg⁻¹ given intravenously (i.v.)) at 8 h and 1 h before challenge, then inoculated with 120 tissue culture infectious doses (TCID₅₀s) of HIV-1 IIIB (30 chimp infectious doses). After challenge, the animals received further CD4-IgG treatment for 9 weeks. A control animal was similarly challenged but not treated. This dose of HIV-1 IIIB is expected to result in seroconversion of all untreated animals within 9 weeks (see, for example, ref. 12). The serum concentrations of CD4-IgG were predicted from the known human pharmacokinetics and checked by enzyme-linked immunosorbent assay (ELISA) where indicated (Fig. 1). The serum concentrations of CD4-IgG attained during the first 2 days of treatment were estimated to be 50–200 µg ml⁻¹, and over the next 5 weeks were predicted to be 5–120 µg ml⁻¹; trough concentrations were confirmed by ELISA. When doses were given i.v. (week 6 onwards), the predicted concentrations of 0.5–200 µg ml⁻¹ were confirmed by ELISA. Treated animals were also monitored for the development of antibodies to CD4-IgG by indirect ELISA. Chimpanzee 37 transiently showed just detectable antibody levels at weeks 7–13 (not shown). Chimpanzee 43 had no detectable antibody response.

Chimpanzees were monitored for HIV infection by detection of virus and by analysis for seroconversion. Virus was detected by a viral coculture assay, in which p24 production was measured, and by the polymerase chain reaction (PCR). The development of antibodies to HIV proteins was assessed by western blot and ELISA whole-virus based assays. The control animal became infected 3 weeks after challenge, as shown by viral coculture (Table 1), and virus could be detected by PCR at 11 weeks. Antibody to the viral protein p24 became evident in this animal by western blot at week 7 (Fig. 2), and subsequently antibodies to other viral proteins, including gp120, were detected. The animal showed seroconversion to HIV by ELISA at week 7 (Table 1). By contrast, the CD4-IgG-treated animals have not shown any signs of infection, after 47 weeks. A single sample from animal 37 (week 23) was positive in two of three PCR assays using a *gag* primer pair (Table 1), but negative using a pair of *env*-derived primers of identical sensitivity (Table 1 legend). Further samples from the same animal up to week 47 have repeatedly assayed negative by both PCR and viral culture. It thus seems that both treated animals were protected from infection.

Immunization with recombinant gp120 (ref. 13) or successive immunization with a variety of immunogens including the V3 loop of gp120 (ref. 14) can induce protective immunity in chimpanzees against later challenge with HIV-1 IIIB. But passive protection from HIV infection has not been previously shown. Protection of chimpanzees against HIV-1 by pretreatment with a single high dose of purified hyperimmune gamma-globulin (HIVIG) obtained from AIDS patients has been observed (A.M.P. *et al.*, manuscript in preparation). The control animal described here was shared with the HIVIG study. In our study, treatment with CD4-IgG continued for 9 weeks, in contrast to the single dose of HIVIG. Further work is needed to determine the necessary dose and length of treatment with CD4-IgG.

Azidothymidine (AZT), the only drug currently approved for the treatment of AIDS, has not yet been shown to prevent infection by HIV or simian immunodeficiency virus (SIV) in animal models, whether given before¹⁵ or after¹⁶ virus challenge, although it does delay the spread of infection. It is unclear whether AZT can prevent infection after accidental challenge with HIV or maternal–fetal transfer in humans; AZT failures in both forms of challenge are known^{17,18}. Clinical trials to address these issues are under way.

Although our results, and those of others (A.M.P. *et al.*, manuscript in preparation), show that it is possible to prevent HIV-1 infection by passive transfer of immunity, it is unclear

TABLE 1 Detection of HIV infection

Week	ELISA	Animal 62 (control) p24	PCR	ELISA	Animal 37 (CD4-IgG) p24	PCR	ELISA	Animal 43 (CD4-IgG) p24	PCR
0	<400	—	ND	<400	—	ND	<400	—	ND
1	<400	—	—	<400	—	—	<400	—	—
2	<400	—	—	<400	—	—	<400	—	—
3	<400	+	—	<400	—	—	<400	—	—
5	<400	+	ND	<400	—	ND	<400	—	ND
7	400	+	ND	<400	—	—	<400	—	—
9	6400	+	ND	<400	—	ND	<400	—	ND
11	3200	+	+	<400	—	—	<400	—	—
13	>6400	+	+	<400	—	—	<400	—	—
15	6400	+	+	<400	—	—	<400	—	—
19	12800	+	+	<400	—	—	<400	—	—
23	12800	+	+	<400	—	+/—	<400	—	—
27	12800	+	+	<400	—	—	<400	—	—
31	12800	+	+	<400	—	—	<400	—	—
35	>6400	+	+	<400	—	—	<400	—	—
39	12800	+	+	<400	—	—	<400	—	—
43	>6400	+	+	<400	—	—	<400	—	—
47	>6400	+	+	<400	—	—	<400	—	—

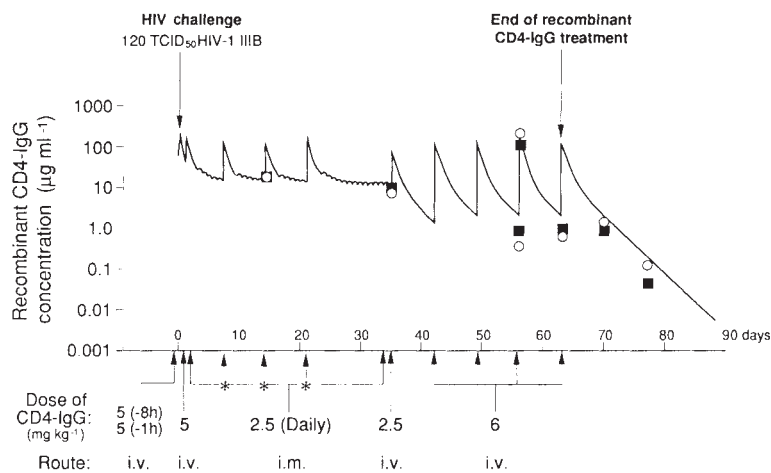
Detection of HIV infection by antibody ELISA and viral culture. Antibody titres against HIV-1 (first column) were determined using a commercial liquid-phase ELISA (Genetics Systems). Cocultivation of HIV was done as described²² for 4 weeks and infection was detected by assaying for viral p24 antigen (second column). For PCR analysis (third column), DNA was extracted from peripheral blood mononuclear cells using digestion buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 1% Tween-20, 1% Nonidet P-40 and 120 µg ml⁻¹ proteinase K). Lysate was incubated at 60 °C for 1 h and extracted with phenol and chloroform. The resulting DNA was solubilized in 25 mM Tris-HCl, pH 7.5. PCR was done essentially as described by Saiki *et al.*²³ for 35 cycles, using the SK 38/39 primer pair, which is specific for the nucleotide sequence of the p24 core protein. Ten copies of proviral DNA can be detected in this assay, based on titration using the Perkin-Elmer HIV-1 PCR kit. Liquid hybridization was done to detect positive samples. Amplified sample (10 µl) was incubated with 1.25 ng ³²P-labelled SK19 probe (specific activity 10⁷ µg⁻¹) and 1.25 µl ReACT buffer at 95 °C for 10 min and snap-cooled. The tubes were incubated at 56 °C for 30 min and centrifuged briefly, loading buffer was added and the samples were applied to a 12% polyacrylamide gel. Positive samples were detected by autoradiography. The sample from animal 37 (week 23) was also subjected to PCR analysis using primer pairs SK68/69, detected by hybridization with probe SK70, with negative results. DNA (1–3 µg) in 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.032 mM each dNTP, 50 ng each primer and 1 unit *Taq* polymerase was amplified 40 times in a cycle consisting of 94 °C, 1 min; 56 °C, 1 min; 72 °C, 1.5 min. The sensitivity of this PCR reaction is identical to that of the SK38/39 pair described above (10 copies).

ND, not determined.

whether CD4-IgG itself will be clinically effective in this mode. It will be important to test the ability of CD4-IgG to protect against HIV-1 infection using different strains of HIV-1, as some primary patient isolates of HIV-1 are considerably less sensitive than HIV-1 IIIB to inhibition by CD4 derivatives¹⁹. Also, the challenge was given as cell-free virus; cell-associated virus may

be more difficult to inhibit. Nevertheless, these results offer hope for the use of CD4-IgG in the prevention of HIV infection in unborn infants. Currently 15–45% of the infants born to HIV-infected women are themselves infected (see, for example, ref. 22), and no drug is known to decrease transmission. Although there is as yet no definitive information on the time at which

FIG. 1 Predicted blood concentrations of CD4-IgG over the course of treatment. CD4-IgG (5 mg per kg bodyweight) was given i.v. for the first three doses (–8, –1 and +24 h postchallenge). For the following 5 weeks, daily injections of 2.5 mg kg⁻¹ CD4-IgG were given intramuscularly (i.m.). On days when the animals were anaesthetized for blood sampling (days 7, 14 and 21), a 5 mg kg⁻¹ dose was given i.v. (*); on day 35 a 2.5 mg kg⁻¹ dose was given i.v. In weeks 6–9, an i.v. dose of 6 mg kg⁻¹ was given once per week to reduce the stress on the animals. Weights for chimpanzees 37 and 43 were 63 and 43 kg, respectively. The concentration of CD4-IgG was estimated by assuming that chimpanzee pharmacokinetics closely mimic those observed in humans (T. Hodges, J. D. Allan, J. Kahn and J. Groopman, personal communication), as the body weights are close. For i.v. administration, the CD4-IgG serum concentrations (*C*) were predicted by dose-adjusting the pharmacokinetics observed in humans following 1 mg kg⁻¹ i.v. bolus injection, which are given by $C(\mu\text{g ml}^{-1}) = 17.5 e^{(-0.059t)} + 3.1 e^{(-0.014t)}$, where time (*t*) is in hours, using a multiple dose simulation routine²¹. For i.m. administration, a 9-h adsorption half-life and 25% bioavailability were assumed. Squares and circles show serum concentrations of CD4-IgG in chimpanzees 43 and 37, respectively, as measured in a double-sandwich ELISA using two monoclonal antibodies to the CD4 portion of the molecule. The first antibody was passively adsorbed into 96-well microtitre plates in NaHCO₃ buffer (0.05M), pH 9.6, overnight at 4 °C. After washing 3 times with PBS containing 0.05% Tween-20 (wash buffer), the antibody was blocked with PBS containing 0.5% BSA and 0.05% Tween



20 for 1 h at room temperature and washed again. Standards, controls and samples diluted in primate serum diluent were added for 2 h at room temperature. After washing 3 times in wash buffer, the second antibody, conjugated to horseradish peroxidase, was added for 1 h at room temperature. Orthophenylenediamine was used as a substrate and plates were read by absorbance at 492 nm after ~30 min.

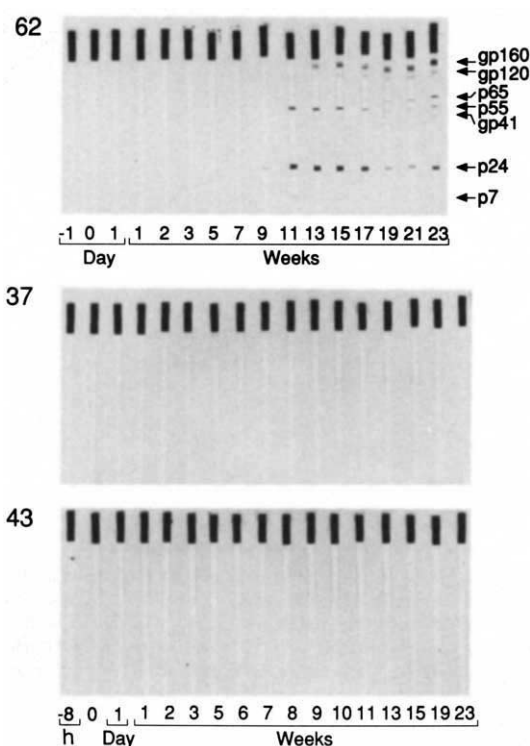


FIG. 2 Western blot seroanalysis of treated (numbers 37 and 43) and control (no. 62) chimps. Samples of serum taken at different times during the protocol were diluted and incubated with commercial (Bio-Rad) western blot strips. The strips were incubated with alkaline phosphate-coupled anti-human IgG (Cappel) and developed using a phosphatase substrate system (Kirkegaard and Perry Laboratories) under conditions recommended by the manufacturers. Samples up to week 47 post-challenge gave identical results (not shown).

fetal infection occurs, an analogy with hepatitis B infection would suggest that most infection occurs late in the third trimester of pregnancy or around the time of delivery. CD4-IgG, like the parent IgG molecule, is transported across the placental barrier during pregnancy in a rhesus monkey model⁹. Thus it may be possible to provide a protective level of CD4-IgG in the fetus before infection with HIV occurs. □

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Prevention of HIV-2 and SIV_{sm} infection by passive immunization in cynomolgus monkeys

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INFECTION of macaques with simian immunodeficiency virus (SIV)^{1,2} and human immunodeficiency virus type 2 (HIV-2)^{3,4} are useful models for studies of immunotherapy and vaccination against HIV as well as for testing of antiviral drugs. Vaccine research showing protective immunity in immunized monkeys⁴⁻¹⁰ has indicated that it will be possible to develop a vaccine for prevention of human HIV infection, although many hurdles remain. The design of an HIV vaccine would be helped if the basis of the protective immunity could be elucidated. Passive immune prophylaxis offers a means to determine the relative role of antibodies in protection against infection. We have studied whether a transfer of antibodies can prevent HIV-2 and SIV_{sm} (SIV of sooty mangabey origin) infection in cynomolgus monkeys. Sera with high antibody titres were collected, heat-treated and injected into naive animals 6 h before challenge with 10-100 monkey-infectious doses of live homologous virus. All control animals treated with normal monkey serum ($n = 6$) or no serum ($n = 39$) became infected by the challenge virus, whereas five out of seven animals pretreated with antibody-containing serum at a dose of 9 ml kg⁻¹ resisted infection. Thus passively transferred antibodies can protect against a low-dose lentivirus challenge in a nonhuman primate.

Two anti-HIV-2 serum pools were obtained from a cynomolgus monkey (A5) (*Macaca fascicularis*) which earlier had been immunized with an inactivated whole-virus vaccine and protected against a homologous HIV-2 challenge⁴. This monkey (A5) received five intramuscular 100 µg doses of whole, inactivated HIV-2_{SBL-6669} emulsified in incomplete Freund's adjuvant on

TABLE 1 Antibody titres in serum pools used for passive immunization of cynomolgus monkeys

Sample	Titres of antibodies in:		
	Whole antigen ELISA	V3 peptide ELISA	Neutralization assay
Anti-HIV-2 serum pool 1	70,000	1,100	1,280
Anti-HIV-2 serum pool 2	270,000	4,800	2,560
Anti-SIV _{sm} serum pool	58,000	10,000	80

The serum pools had antibody reactivity to *gag-pol*- and *env*-encoded structural proteins as detected by immunoblotting using antigen preparation of HIV-2_{SBL-6669} (data not shown). The antibody content of serum pools and passively immunized animals was determined in whole HIV-2_{SBL-6669} viral lysate ELISA¹⁵ and in homologous V3 peptide ELISA¹⁶. The V3 peptide assay was included for antibody determination because a recent study¹⁷ has demonstrated immunogenic dominating linear sites of HIV-2_{SBL-6669} with capacity to induce neutralizing antibodies and cytotoxic antibodies in this region. The neutralizing antibodies were determined by incubation of twofold dilutions of serum and a stock virus preparation of HIV-2_{SBL-6669} or SIV_{sm} before addition of HUT-78 cells. The supernatants were analysed in an HIV-2/SIV antigen assay¹⁸ after 7 days in culture. The neutralizing titre was defined as the reciprocal of the highest dilution giving a 50% reduction in absorbance value in the antigen assay. Sera collected from the monkeys before passive immunization were used as negative controls in the serological assays.

TABLE 2 Antibody titres and results of virus isolation and PCR in cynomolgus monkeys passively immunized by anti-HIV-2 sera and challenged with 10 MID₅₀ of HIV-2_{SBL-6669/H5} at day 0

Monkey	Antiserum used	Dose of serum	Titres of antibodies in:			Presence of virus at day:							
			Whole antigen ELISA	V3 peptide ELISA	Neutralization assay								
						0	14	21	30	45	60	90	133
B21	Anti-HIV-2 (pool 1)	3 ml kg ⁻¹	7,000	100	40	-	+	+	+	+	+	-	-(+)
B22	Anti-HIV-2 (pool 1)	3 ml kg ⁻¹	6,000	100	80	-	-	-	-	-	-	-	-(+)
B23	Anti-HIV-2 (pool 1)	3 ml kg ⁻¹	7,000	100	20	-	-	+	+	+	+	-	-(+)
B24	Anti-HIV-2 (pool 1)	3 ml kg ⁻¹	7,200	100	20	-	+	+	+	+	+	+	+(+)
S1	Normal serum	3 ml kg ⁻¹	<100	<20	<10	-	+	+	+	+	+	+	+(+)
S2	Normal serum	3 ml kg ⁻¹	<100	<20	<10	-	+	+	+	+	+	-	-(+)
S3	No serum		<100	<20	<10	-	+	+	+	+	+	-	-(+)
S4	No serum		<100	<20	<10	-	+	+	+	+	-	-	-(+)
B51	Anti-HIV-2 (pool 2)	9 ml kg ⁻¹	38,000	500	320	-	-	+(+)	+(+)	+(+)	-(+)	-(+)	-(+)
B52	Anti-HIV-2 (pool 2)	9 ml kg ⁻¹	24,000	350	320	-	-	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)
B6	Anti-HIV-2 (pool 2)	9 ml kg ⁻¹	29,000	500	320	-	-	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)
B5	Normal serum	9 ml kg ⁻¹	<100	<20	<20	-	+	+(+)	+(+)	+(+)	+(+)	-(+)	-(+)
PH1	Normal serum	9 ml kg ⁻¹	<100	<20	<20	-	+	+(+)	+(+)	+(+)	+(+)	+(+)	-(+)
H39	No serum		<100	<20	<20	-	+	+(+)	+(+)	+(+)	+(+)	-(+)	-(+)

HIV-2_{SBL-6669/H5} was previously titred *in vivo* in monkeys as described⁴. Virus isolations were performed as previously described¹⁹, with one important improvement. The culture supernatants were screened by a sensitive HIV-2/SIV antigen assay¹⁸, in addition to the previously used reverse transcriptase assay. PCR with nested primers was done essentially as previously described²⁰, but a different lysis buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA, 0.5% NP40, 0.5% Tween-20) and a higher primer annealing temperature (60 °C) was used. The primers were derived from HIV-2/SIV consensus sequences (Los Alamos database) and are given with their positions in HIV-2_{SY} (strain SBL-6669) indicated in parenthesis (+, sense; -, antisense): HIV-2/SIV *gag*, JA47 (TGGTGCATGCACGACAGAGAGAAAG (+, 802)), JA48 (CAAGTAGACCAACAGCACCACCTAG (+, 926)), JA49 (CCTGAAATCCTGGCACTACTTCTGC (-, 1048)) and JA52 (ATATCATAGGGCGTGCACCTTCTG (-, 1079)), HIV-2/SIV *pol*, JA43 (AAGCCAAGGAGTAGTGAAGCAATG (+, 4490)), JA44 (GAAGGGGAGGAATAGGGGAT-ATGAC (+, 4612)), JA45 (CCACAGCTGATCTCTGCTTCTCTG (-, 4733)), and JA46 (TCTGTCCCTACCTTTACGATGACTG (-, 4795)).

+, Virus isolated; (+), virus DNA detected by PCR.

days 0, 35, 56, 77 and 734 and was challenged 2 weeks later with 100 monkey-infectious doses (MID₅₀) of HIV-2_{SBL-6669/H5}. Serum samples were obtained 120–189 days after challenge (pool 1). The animal then received a 100 µg booster injection of killed-virus vaccine on day 300 (after challenge) and a second serum pool was collected 3–5 weeks after the final boost (pool 2).

An anti-SIV_{sm} serum pool was obtained from a cynomolgus monkey (H56) which has remained clinically healthy with normal CD4 counts during more than one year of experimental infection with SIV_{sm}. Monkey H56 had been inoculated with 1–10 MID₅₀ of SIV_{sm} (see legend to Table 3) and serum samples were collected 220–371 days postinoculation.

The serum pools were heat-inactivated (56 °C for 30 min) before administration into animals. Antibody titres of the serum

pools are shown in Table 1. The serum pools were free from infectious virus because two naive cynomolgus monkeys that received 20 ml of the heat-treated anti-SIV serum intravenously, were repeatedly negative by virus isolation and by polymerase chain reaction (PCR) analysis and showed declining anti-SIV antibody titres (data not shown).

Protection against HIV-2 and SIV infection was assessed by virus isolation and PCR on monkey peripheral blood mononuclear cells as well as by antigen and antibody assays. In one experiment, four monkeys received a low dose (3 ml kg⁻¹ body weight from pool 1) and three animals a high dose (9 ml kg⁻¹ from pool 2) of anti-HIV-2 serum 6 h before challenge with 10 MID₅₀ of HIV-2_{SBL-6669/H5} (ref. 4). Seven control animals received either normal monkey serum (*n* = 4) or no serum (*n* = 3) and were exposed to the same virus dose. Protection against the

TABLE 3 Antibody titres and results of virus isolation and PCR in cynomolgus monkeys passively immunized by anti-SIV_{sm} sera and challenged with 10–100 MID₅₀ of SIV_{sm} at day 0

Monkey	Antiserum used	Dose of serum	Titres of antibodies in:			Presence of virus at day:							
			Whole antigen ELISA	V3 peptide ELISA	Neutralization assay								
						0	14*	30	45	60	90	120	165
B31	Anti-SIV	9 ml kg ⁻¹	4,200	1,000	20	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)
B32	Anti-SIV	9 ml kg ⁻¹	6,000	1,000	20	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)
B33	Anti-SIV	9 ml kg ⁻¹	5,000	1,100	10	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)	-(+)
B34	Anti-SIV	9 ml kg ⁻¹	3,700	500	10	-(+)	+(+)	+(+)	+(+)	+(+)	-(+)	-(+)	-(+)
B35	Normal serum	9 ml kg ⁻¹	<100	<20	<10	-(+)	+(+)	+(+)	+(+)	+(+)	-(+)	+(+)	+(+)
B36	Normal serum	9 ml kg ⁻¹	<100	<20	<10	-(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	-(+)

Multiple aliquots of a cell-free culture supernatant of SIV_{sm} (SMM-3)-infected PHA-stimulated human PBMC were stored at -70 °C. The minimum infectious dose of this stock was determined by titration in 14 cynomolgus monkeys using two animals for each dilutions. Tenfold dilutions of virus pool from 1 to 10⁻⁷ were inoculated intravenously at 1 ml per animal. Infection was monitored by virus isolation and seroconversion as described²³. All animals receiving doses of virus ranging from 1 to 10⁻⁵ became infected, whereas none of the monkeys that received higher dilutions of virus (10⁻⁶–10⁻⁷) became infected. It was concluded that the virus stock employed contains about 10⁵–10⁶ MID₅₀ doses per ml.

+, Virus isolated; (+), virus DNA detected by PCR.

* Presence of viral antigen in serum was demonstrated, as previously described¹⁷, 14 days after challenge with SIV_{sm} in the two control animals but in none of the passively immunized monkeys.

homologous HIV-2 challenge was obtained in one out of four animals that received the low-serum dose and in two of three animals that received the higher serum dose (Table 2). All seven controls as well as 27 monkeys in separate experiments became infected with the same HIV-2 challenge dose.

In a second experiment, four cynomolgus monkeys were given 9 ml kg⁻¹ anti-SIV_{sm} serum and 6 h later challenged with 10–100 MID₅₀ of SIV_{sm}. Two control animals received 20 ml normal monkey serum before challenge. Three out of four monkeys pretreated with SIV_{sm} antiserum showed no signs of infection following a homologous SIV_{sm} challenge, whereas the two controls became infected (Table 3). In another experiment (not shown), nine cynomolgus monkeys were inoculated with the same dose, 10–100 MID₅₀ of SIV_{sm}, and all nine monkeys became infected.

Antibody titres as determined with whole-antigen enzyme-linked immunosorbent assay (ELISA) declined after challenge with a half-life of approximately 7–9 days and became undetectable at 3–4 months in protected animals (monkeys B22, B52, B6 and B31–33). This further indicates that active infection did not occur during 6–10 months of follow-up after live virus challenge. Five out of seven monkeys treated with 9 ml kg⁻¹ of

anti-HIV-2 or anti-SIV serum resisted the homologous cell-free virus challenge, whereas a lower dose of anti-serum appeared to be less efficient. Our data suggest that the titre of viral specific antibodies may be critical for protection, but the prechallenge antibody titres in individual monkeys did not show a clear correlation with protection. Larger studies will be required to establish both the amount and quality of antibodies that give protection.

Successful passive immunization against primate lentiviruses in a nonhuman primate has to our knowledge not been reported previously. Prince *et al.*¹¹ reported that passive transfer of pooled IgG from HIV-1-infected humans failed to protect chimpanzees against live HIV-1 virus challenge. But passive transfer of anti-envelope antibodies has been shown to protect against murine and feline oncogenic retroviruses^{12,13} and recently Kataoka *et al.* showed that human antibodies to human T-cell leukaemia virus type I (HTLV-I) prevented HTLV-I infection in rabbits¹⁴. We have shown here that antibodies alone are sufficient to protect against HIV-2 and SIV infection in a nonhuman primate. Studies on post-exposure prophylaxis and protection against heterologous virus strains are in progress. □

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Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions

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THE self-renewal and differentiation of haematopoietic stem cells occurs *in vivo* and *in vitro* in direct contact with cells making up the haematopoietic microenvironment^{1–4}. In this study we used adhesive ligands and blocking antibodies to identify stromal cell-derived extracellular matrix proteins involved in promoting attachment of murine haematopoietic stem cells. Here we report that day-12 colony-forming-unit spleen (CFU-S₁₂)⁵ cells and reconstituting haematopoietic stem cells attach to the C-terminal, heparin-binding fragment of fibronectin by recognizing the CS-1 peptide of the alternatively spliced non-type III connecting segment (IIICS) of human plasma fibronectin. Furthermore, CFU-S₁₂ stem cells express the α_4 subunit of the VLA-4 integrin receptor, which is known to be a receptor for the CS-1 sequence, and monoclonal antibodies against the integrin α_4 subunit of VLA-4 block adhesion

of CFU-S₁₂ stem cells to plates coated with the C-terminal fibronectin fragment. Finally, polyclonal antibodies against the integrin β_1 subunit of VLA-4 inhibit the formation of CFU-S₁₂-derived spleen colonies and medullary haematopoiesis *in vivo* following intravenous infusion of antibody-treated bone marrow cells.

One role of stromal cells making up the haematopoietic microenvironment in the maintenance of haematopoiesis may involve secretion of extracellular matrix (ECM) proteins to provide anchorage sites for colocalization of stem cells and growth factors^{6–8}. To examine the role of ECM in haematopoietic stem-cell adhesion, we first analysed the binding of CFU-S₁₂ by adherence depletion assays in which a limiting dilution of freshly isolated bone marrow cells was incubated on ECM produced by a murine bone marrow-derived stromal cell line, U2, capable of replacing the haematopoietic microenvironment of a long-term marrow culture in supporting haematopoiesis *in vitro*^{9,10}. After a 2-h incubation at 37 °C, the nonadherent population of cells was collected and assayed for CFU-S₁₂. Incubation of bone-marrow cells on U2 ECM resulted in the depletion of 50% of CFU-S₁₂ compared with cells incubated on gelatin-coated dishes (control) (11.7 ± 1.3 versus 24.3 ± 3.1 nonadherent CFU-S₁₂; Table 1). To determine the long-term haematopoietic reconstituting capacity of U2 ECM-bound cells, a larger inoculum of bone marrow cells obtained from male mice was incubated on the U2 ECM. After careful removal of nonadherent cells, the bone marrow cells adherent to U2 ECM were collected and injected into lethally irradiated female mice. Southern blot analysis of DNA isolated from thymus, bone marrow and spleen cells from these female mice 4 months post-transplant revealed a prominent Y chromosome-specific hybridizing band (Fig. 1a), demonstrating that cells adhering to U2 ECM not only contain CFU-S₁₂, but are also capable of multilineage and long-term

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reconstitution of irradiated animals.

Although stromal cell ECM (including U2) is composed of collagens, laminin, and fibronectin^{9,11}, we found that neither collagen types I, III and IV, nor laminin promoted significant attachment of CFU-S₁₂ (data not shown). As primitive erythroid progenitors (K. Goltry and V. Patel, manuscript in preparation) and lymphoid cells¹²⁻¹⁴ attach specifically to the C-terminal, heparin-binding fragments of fibronectin, we analysed stem cell binding to dishes coated with intact fibronectin or its chymotryptic fragments. Incubation of bone marrow cells on bacteriological plates coated with a C-terminal heparin-binding fragment of fibronectin (HBD) of relative molecular mass 30,000-35,000 (*M_r* 30-35K) resulted in 55% reduction in the number of nonadherent CFU-S₁₂ (Table 1). As in previous studies of stem cells¹⁵ and primitive BFU-E (K. Goltry and V. Patel, manuscript in preparation), no adherence of CFU-S₁₂ was demonstrated on intact fibronectin (Table 1). Furthermore, no appreciable adherence of CFU-S₁₂ was detected on a 115K cell-binding domain containing the RGDS sequence (Table 1), or a 42K C-terminal fibronectin fragment containing the HBD, but not a second cell-binding domain located in this same region of fibronectin

TABLE 1 Analysis of CFU-S adhesion to U2 stromal cell ECM and cell adhesion domains of fibronectin

Substrates	Nonadherent CFU-S	Depletion (%)
Control	24.3 ± 3.1*	0
ECM	11.7 ± 1.3	50
ECM + heparin†	8.5 ± 2.5	65
Control	40.5 ± 2.9*	0
Fibronectin	41.0 ± 1.3	0
HBD (30-35K)	18.0 ± 8.4	55
CBD (115K)	45.0 ± 4.7	0
Control	8.0 ± 2.6‡	0
CS-1/BSA	4.0 ± 3.5	50
CS-3/BSA	7.8 ± 2.7	0
Control	6.7 ± 1.3§	0
HBD (30-35K)	2.7 ± 1.3	60
HBD (30-35K) + αH2Ab	3.4 ± 2.4	49
HBD (30-35K) + LPAM-1 Ab	7.0 ± 1.1	0

Results shown are representative experiments. The data are expressed as mean ± s.d. of at least four animals per experiment. Bone marrow cells were collected as described¹⁰ from male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine). Extracellular matrix was prepared as previously described³² after growing U2 cells on tissue culture plates (either 6-well or 10-cm; Corning, New York) to confluence. In some experiments, ECM was treated for 30 min with 500 µg heparin sulphate (Sigma) in phosphate-buffered saline (PBS) per 60-mm dish. Plates were then washed free of unbound heparin with PBS before addition of cells. For CFU-S₁₂ adherence, limiting dilutions (1-3 × 10⁵) of plastic adherence-depleted bone marrow cells were then placed on U2, U2 ECM (plates coated with 0.1% gelatin (Sigma) were controls for nonspecific binding of CFU-S₁₂ in the ECM-binding experiments) or bacteriologic plates coated with protein fragments (see below). For attachment assays to fibronectin fragments, 35-mm bacteriologic petri dishes (Falcon, Lincoln Park, New Jersey) were coated with 2 ml PBS⁻ solutions containing 20 µg ml⁻¹ indicated substrates or 2% BSA (control), as previously described³³. For attachment assays to synthetic peptides, 24-well nontissue culture plates (Costar, Cambridge, Massachusetts) which had been coated with 250 µg per well BSA or BSA conjugate (50-60 mol peptide per mol BSA) were used. After a 2-h incubation at 37 °C in 5% CO₂, nonadherent cells were collected and saved. For blocking studies, bone marrow cells (2.5 × 10⁵ ml⁻¹) were preincubated at 4 °C with 16 µg ml⁻¹ of LPAM-1 (or rat anti-H2 pan monoclonal antibodies-M1/42.3.9.8.HLK, 1:100 dilution)²⁴ for 30 min rotating end-over-end. Antibody-treated cells (1.25 × 10⁵) were plated in 0.5 ml at 4 °C in 24-well bacteriologic plates which had been precoated with 30-35K fibronectin fragment (or 2% BSA) as above. After a 2 h incubation, nonadherent cells were carefully collected, trypsinized for 1 min at 37 °C to remove remaining antibody. All nonadherent cells were collected by three gentle washes with warmed media containing 2% BSA and all washes were combined and injected into lethally irradiated mice (1,250 rads, split dose with 3 h between doses at 145 rads per min; this dose of irradiation is lethal to 100% of mice not receiving bone marrow infusions). Spleens collected 12-14 days post-transplant were fixed and surface colonies counted as described¹⁰.

* Nonadherent CFU-S after overlaying 3 × 10⁵ cells for 2 h on ECM or protein-coated plates. Control was 0.1% gelatin or 2% BSA-coated plates.

† ECM was preincubated with 500 µg heparin sulphate per 60-mm dish before incubation of cells, see legend.

‡ Nonadherent CFU-S after overlaying 9 × 10⁴ cells for 2 h on peptide-coated plates. Control, BSA-coated plates.

§ Nonadherent CFU-S after overlaying 1.25 × 10⁵ antibody-treated cells for 2 h on peptide-coated plates. Control, BSA-coated plates.

called the CS-1 sequence (data not shown). The lack of adherence to the 42K fragment was in agreement with our findings that preincubation of U2 ECM with heparin sulphate had minimal effects on CFU-S₁₂ adherence (Table 1). These data suggest little, if any, adhesion of CFU-S₁₂ to the heparin-binding site (II) (ref. 16) located in both the 42K and the 30-35K fragments and implicate the CS-1 sequence¹⁷⁻¹⁹ in this adherence.

This possibility was examined by measuring adhesion of CFU-S₁₂ to bacteriological plates coated with synthetic CS-1 peptide covalently crosslinked to bovine serum albumin (BSA). Incubation of bone marrow cells on CS-1/BSA resulted in 50% reduction of nonadherent CFU-S₁₂ compared to bone marrow cells incubated on plates coated with BSA alone or CS-3/BSA conjugate (control synthetic peptide present in IIICS amino-acid sequence) (Table 1). The presence of CS-1 sequence in the 35K fragment of fibronectin was confirmed by immunoblotting with anti-CS-1 peptide antibody, and the presence of CS-1 containing differentially spliced messenger RNA in U2 stromal cells was confirmed by northern blot analysis using a synthetic 75-base oligonucleotide probe representing the CS-1 sequence of rat fibronectin (ref. 20; R. S. Dwivedi, V.P.P. and D.A.W., unpublished data). Adherence of CFU-S₁₂ to the 30-35K fibronectin fragment was dose-dependent and saturable (data not shown). CFU-S₁₂ that were nonadherent to optimal concentrations of 30-35K fragment gave rise to fewer multilineage spleen colonies (1 out of 15 colonies, or 6.7%) when compared with control injections (12 out of 28 colonies, or 42.9%), demonstrating that the adherence to this fragment defines a more primitive CFU-S compartment. Haematopoietic stem cells (from male mice) adherent to the 30-35K domain were also capable of multilineage haematopoietic reconstitution of irradiated female mice (Fig. 1b), demonstrating that cells adherent to this fragment include long-term reconstituting stem cells.

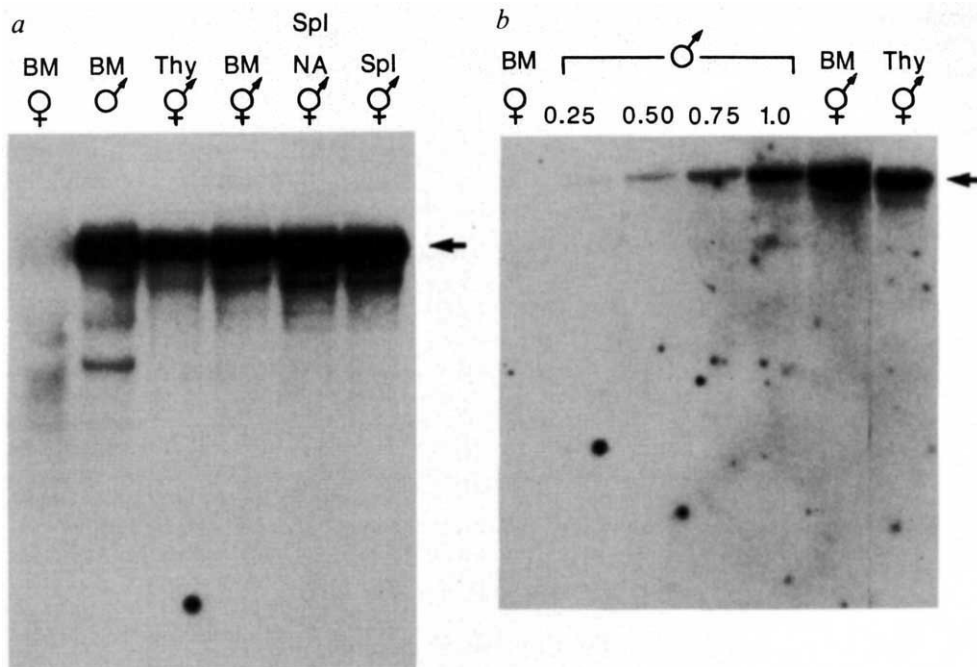
Cell adhesion to ECM proteins is mediated by the integrin family of cell-surface receptors²¹. Adhesion of some lymphoid cells to the CS-1 sequence of fibronectin is mediated by the VLA-4 integrin, a heterodimer consisting of α₄ and β₁ subunits¹⁷⁻¹⁹ and this α₄-subunit is apparently shared by the

TABLE 2 Expression of VLA-4 on CFU-S

Primary antibody	Number of cells injected	Spleen colonies	CFU-S content
Presort	2 × 10 ⁵	10.3 ± 0.8	5.0 per 10 ⁵
LPAM-1	0.9 × 10 ⁵	6.2 ± 2.2	6.9 per 10 ⁵
Mac-1	2 × 10 ⁵	0	<0.5 per 10 ⁵

Plastic adherence-depleted cells and low-density bone marrow mononuclear cells isolated by centrifugation on a Ficoll-hypaque gradient (Histopaque-1119, Sigma) (10⁶ ml⁻¹) were incubated with undiluted antibodies to VLA α₄ (murine anti-α₄ monoclonal-LPAM-1)²², 1:50 dilution of rat anti-murine Mac-1 monoclonal antibody²³ (Boehringer Mannheim) or in α minimum essential medium (MEM) (Gibco, Grand Island, New York) containing 5% fetal calf serum alone at 4 °C for 45 min while rotating end over end. Cells were then washed twice with ice-cold PBS and treated with a 1:100 dilution of FITC-labelled goat anti-rat or goat anti-mouse IgG (Tago, Burlingame, California) for 45 min at 4 °C. After incubation with the secondary antibody the cells were washed twice with ice cold PBS and resuspended in α MEM containing 5% fetal calf serum at 1-3 × 10⁶ cells per ml for cell sorting. Fluorescein-labelled cells were sorted on a Cytofluorograf II (Ortho Diagnostics) containing an Argon laser and band filter for 510-540 nm wavelengths using gates selected for medium cell size (8-10 µm) based on forward and 90° light scatter. Positive cells were selected on the basis of fluorescence intensity that was >2 s.d. above the mean channel of negative control cells (unstained by first antibody) and 36% of positive cells exhibiting medium bright fluorescence were collected on ice, washed once with media containing 5% fetal calf serum, trypsinized for 2 min at 37 °C, washed twice in ice-cold medium containing 5% fetal calf serum, and injected into lethally irradiated syngeneic recipients. Data presented were obtained from one of two independent experiments showing similar results.

FIG. 1 Southern blot analysis of female mice transplanted with male bone marrow cells adherent to U2 ECM (a) or 30–35K (b) chymotryptic fragment of fibronectin. For reconstitution assays on ECM (a) (3×10^6) bone marrow cells depleted of plastic adherent cells were plated onto 10-cm tissue culture dishes (Corning) containing cell-free U2 ECM and allowed to adhere for 2 h. The adherent cells were then injected into lethally irradiated female mice (C3H/HeJ, Jackson Laboratories). Four months after transplantation, DNA was prepared from thymus, spleen and bone marrow cells, cut with *Bam*HI, size fractionated on a 1% agarose gel, transferred to a nylon filter (Magnagraph, Micron Separations, Westboro, Massachusetts) and probed with 32 P-labelled Y chromosome-specific probe, PY-2 (ref. 34). Lanes are labelled with the source of cells used for the DNA preparation; BM♀, female bone marrow (negative control); BM♂, male bone marrow (positive control); Thy♂, thymus; BM♂, bone marrow; Spl NA♀, non-adherent spleen cells; Spl♀, total spleen cells all from a female mouse transplanted with male cells adherent to U2 ECM. Arrow, major male-hybridizing band running at 14 kilobases. The figure shows tissues of one representative mouse of a group of three. For reconstitution assays on fibronectin fragments (b), bone-marrow cells were plated onto 30-mm bacteriological dishes (Falcon) coated with 40 μ g per dish of 30–35K chymotryptic fragment of fibronectin (containing CS-1), and adherent cells



collected and transplanted, as described above. Four months after transplant, tissues were collected from mice, DNA prepared and Southern blots performed as in a, except additional controls for copy number were included by preparing DNA from mixtures of male and female bone marrow to represent 25, 50 and 75% male cells. Results from a representative transplant recipient of a group of three mice.

Peyers' patch-specific lymphocyte homing receptor²². We used a monoclonal antibody to murine α_4 , LPAM-1 (ref. 22) and fluorescence-activated cell sorting (FACS), to determine whether CFU-S₁₂ express this subunit of VLA-4. About 36% of bone marrow cells stain positive with LPAM-1 (data not shown). Injection of VLA-4-positive bone marrow cells (collected from a gate representing the middle 33% of fluorescence intensity) gave rise to CFU-S₁₂-derived spleen colonies in lethally irradiated mice (Table 2). By contrast, bone marrow sorted for the presence of Mac-1 antigen²³, which is expressed on more differentiated myeloid cells, failed to form spleen colonies when injected at concentrations 2–5 times higher than LPAM-1-positive cells (Table 2). The expression of the α_4 subunit was further shown to be functionally important as incubation of bone marrow cells with LPAM-1 antibody blocked attachment to 30–35K fibronectin-fragment-coated dishes (Table 1).

Consistent with this result are our observations on the effect of rabbit polyclonal antibodies against the common β_1 -subunit of the VLA integrin receptors on the lodgement of injected stem cells in the spleen and bone marrow *in vivo*. Preincubation of

limiting dilution of bone-marrow cells with intact anti-FnR (β_1)¹⁴ immune IgG or anti-FnR F(ab)₂ fragments resulted in significant reduction in the ability of marrow cells subsequently to give rise to spleen colonies (Fig. 2) or myeloid colonies in the femur (data not shown), whereas control incubations performed in the presence of either preimmune IgG or anti-pan H2 (ref. 24) monoclonal antibody was without significant effect (Fig. 2).

We conclude that the adhesion of primary haematopoietic stem cells to stromal cell ECM is partly promoted by the proteolytic fragments of fibronectin containing the alternatively spliced region of the IIICS domain and we suggest that this interaction is likely to be mediated by the integrin receptor VLA-4 (α_4/β_1). As only one out of three potential products of the alternative splicing of the fibronectin pre-mRNA has been shown to contain CS-1 sequence in the rat²⁰, it will be important to determine the role of stromal cell expression of fibronectin splicing variants and ECM protein remodelling by proteolysis in the previously observed nonrandom, spatial distribution of haematopoietic stem cells in the microenvironment^{25,26}. These

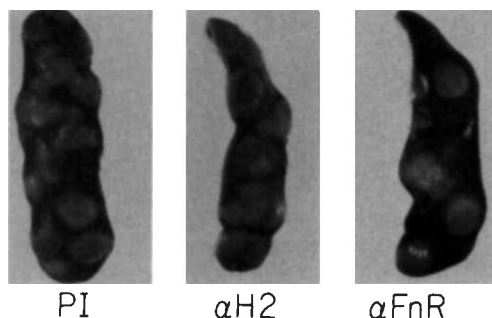


FIG. 2 Inhibition of CFU-S-derived spleen-colony formation by anti-fibronectin receptor antibody. Antibodies used included affinity-purified rabbit anti- β_1 (ref. 14) and F(ab)₂ fragment prepared by pepsin treatment followed by protein A adsorption of Fc-containing antibodies; and rat anti-H₂ pan monoclonal antibodies. Blocking of CFU-S homing to spleen *in vivo* was done by incubating limiting dilution bone marrow cells ($1-3 \times 10^5$ ml⁻¹) depleted of plastic adherent cells with 100 μ g ml⁻¹ rabbit anti-fibronectin receptor (mouse) IgG, 100 μ g ml⁻¹ rabbit preimmune IgG, or 1:50 dilution anti-pan H₂ (as an irrelevant antigen expressed on CFU-S) at 4 °C for 45 min while rotating end over end. After this incubation, cells were washed twice in ice-cold PBS and resuspended in ice-cold α -MEM containing 5% FCS. Cells were kept on ice until injected slowly into lethally irradiated mice. Spleens were harvested 12–14 days post-transplant and analysed as above. Figure shows representative spleens from one experiment.

interactions may have important implications in the localization of intravenously injected stem cells to the medullary cavity during bone marrow transplantation and modulation of expression of VLA-4 may have a role in loss of adhesion of leukaemic blast cells to stromal cells noted during blast crisis in chronic myelogenous leukaemia²⁷. By analogy to lymphocyte homing mechanisms, stem-cell stromal interactions may use

multiple ligand-receptor interactions including VLA-4/CS-1 described here and lectins described previously²⁸. Finally, the recent characterization of the steel (*Sl*) gene product as a transmembrane growth factor strengthens the possibility that an important role of the haematopoietic microenvironment is to provide anchorage sites for stem cells to promote local interactions with membrane-bound cytokines²⁹⁻³¹. □

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A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules

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ASSEMBLY of class I major histocompatibility complex (MHC) molecules involves the interaction of two distinct polypeptides (the heavy and light chains) with peptide antigen. Cell lines synthesizing both chains but expressing low levels of MHC class I molecules on their surface as a result of a failure in assembly and transport have been identified¹. We now report that although the apparent steady-state distribution in these cells of class I molecules is in the endoplasmic reticulum (ER), the molecules in fact are recycled between the ER and Golgi, rather than retained in the ER. This explains the failure of class I molecules to negotiate the secretory pathway. Class I molecules do not seem to be modified by Golgi enzymes, suggesting that the proteins do not reach the Golgi apparatus during recycling. But morphological and subcellular fractionation evidence indicates that they pass through the *cis* Golgi or a Golgi-associated organelle, which we postulate to be the recycling organelle. This compartment, which we call the 'cis-Golgi network', would thereby be a sorting organelle that selects proteins for return to the ER.

We examined the mutant cell lines¹ and failed either to detect carbohydrate processing of class I molecules in the Golgi or to

coprecipitate much light chain with an anti-heavy chain antibody (data not shown). Immunofluorescence staining of these cells with antibodies to class I was consistent with the biochemistry, revealing a typical ER distribution (Fig. 1a)², not a Golgi one as revealed by an antibody to the Golgi marker mannosidase II (man II)³ (Fig. 1b).

The drug brefeldin A induces the rapid redistribution of Golgi proteins into the ER along a pathway that is inhibited by microtubule-disrupting agents, energy poisons and reduced temperatures⁴. We therefore reasoned that the retrograde transport, and hence distribution, of proteins that are normally recycled might also be altered by lowering the temperature or by depolymerizing microtubules. This would enable us to distinguish whether class I molecules are truly retained in the ER or recycled in one of the mutant cell lines, CMT¹. When CMT cells were incubated at 16 °C for 2 h and then warmed for 5 min to 37 °C, class I molecules redistributed to a Golgi-like pattern (Fig. 1c, d), whereas the distribution of the ER marker remained unchanged (not shown). Depletion of ATP with 2-deoxy-D-glucose and sodium azide, which inhibit ATP to Golgi membrane traffic⁶, prevented the change in distribution of class I molecules at 16 °C (not shown). The distribution of a nonresident ER protein, a truncated form of the T-cell-receptor α chain (TCR α_{GPI}), which cannot be secreted but is retained in the ER, was similarly examined. When expressed in Chinese hamster ovary cells kept at 37 °C, TCR α_{GPI} is processed and distributed in the same way as retained MHC class I molecules⁵. Unlike class I, however, TCR α_{GPI} did not assume a Golgi-like distribution at 16 °C (Fig. 1g, h).

Redistribution of class I molecules at 37 °C can be induced using nocodazole, which causes microtubule depolymerization. Within 40 min of nocodazole treatment, class I molecules changed from having a punctate/reticular (ER-like) distribution to having a scattered distribution, corresponding to structures that lacked ER resident proteins, but which often colocalized with the Golgi marker (Fig. 1e, f). The distribution of TCR α_{GPI} did not change after nocodazole treatment.

Class I molecules in cells incubated at 16 °C and then rewarmed to 37 °C (in the presence of cycloheximide to prevent the introduction of newly synthesized class I molecules into the

ER), regained their original steady-state ER distribution (Fig. 1*i,j*; the distribution of the ER and Golgi markers did not change). We, therefore, examined whether this return of class I molecules into the ER followed the characteristics of brefeldin A-induced transport from the Golgi to ER. Class I molecules were first redistributed to and maintained in Golgi-like structures by incubation at 16 °C and then 22 °C. Subsequent treatment

with brefeldin A at 22 °C caused both class I molecules and the Golgi marker to redistribute to the ER. When cells with class I molecules in the Golgi-like structures were rewarmed to 37 °C in the presence of either 2-deoxy-D-glucose and sodium azide or aluminium fluoride, inhibitors of brefeldin A-induced Golgi-to-ER redistribution^{4,7}, class I molecules remained in the Golgi-like structures.

Subcellular fractionation on sucrose density gradients provided further support to these observations. Metabolically labelled class I molecules at 37 °C were distributed mainly in fractions corresponding (from most dense to least dense) to the rough ER, smooth ER and Golgi regions as previously defined⁸. At 16 °C, class I molecules (Fig. 2*a*) were found mainly in a less-dense fraction corresponding to the Golgi region (Fig. 2*b*). The fractions in which the ER and Golgi markers were mainly found remained the same on temperature change (Fig. 2*b*). On

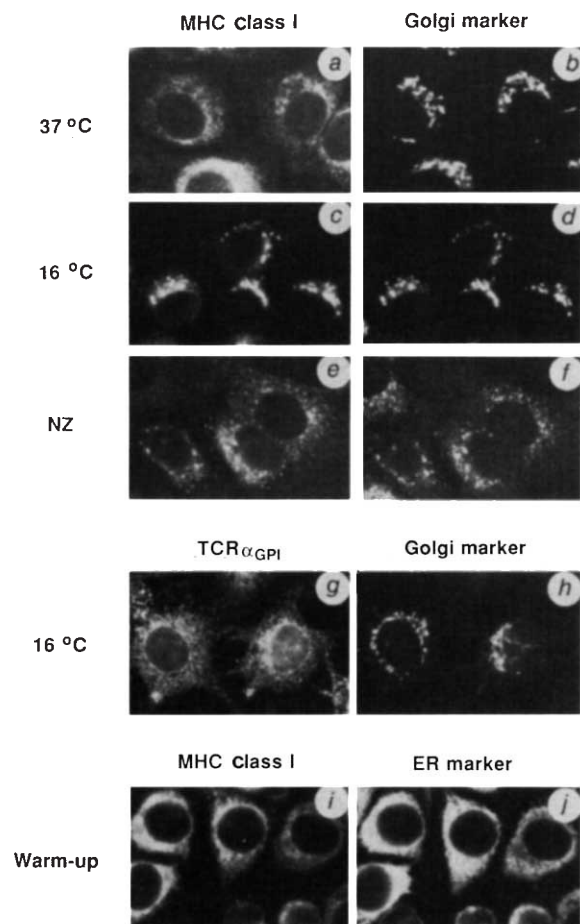


FIG. 1 Immunofluorescent localization of MHC class I and TCR α_{GPI} after temperature change and nocodazole treatment. The distribution of class I molecules (a) at 37 °C was diffuse and ER-like in contrast to the juxtanuclear distribution of the Golgi marker man II (b) in CMT cells double-labelled for these two proteins. At 16 °C, class I (c) redistributed into a Golgi-like pattern (d). After 40 min of nocodazole treatment (20 $\mu\text{g ml}^{-1}$) at 37 °C, class I (e) also largely co-distributed with the Golgi marker (f) which now appeared in Golgi fragments dispersed throughout the cytoplasm. In contrast to MHC class I, TCR α_{GPI} (g) did not redistribute from its ER-like pattern into a juxtanuclear Golgi location (h) at 16 °C in double-labelling experiments. On rewarming cells to 37 °C for 30 min in the presence of cycloheximide, class I molecules re-adopted their (i) original diffuse punctate/reticular distribution which coincided with that of an ER marker (j).

METHODS. CMT cells transfected with the H-2K^d complementary DNA¹ were plated on coverslips and their temperature altered in culture medium supplemented by 10 mM HEPES buffer, pH 7.3, and then fixed, permeabilized and processed as previously described⁴. All 16 °C incubations described in the text were followed by warming in a 37 °C water-bath for 5 min. **Immunofluorescence:** cells on coverslips were incubated with the primary antibodies SF1-1.1.1 (American Type Culture Collection) specific for H-2K^d molecules, anti-ER antiserum (provided by D. Louvard, Pasteur Institute, Paris) specific for ER, and anti-man II antiserum (provided by K. Moremen, Massachusetts Institute of Technology) specific for Golgi, and then by rhodamine-conjugated goat anti-mouse IgG (Cooper Biomedical) for class I, or fluorescein-conjugated goat anti-rabbit IgG (Cooper Biomedical) for ER and Golgi markers. After washing, coverslips were mounted on slides in Fluoromount G (Southern Biotechnology), and viewed with a $\times 63$ oil Planapo lens on a Zeiss microscope with barrier filters to prevent crossover of fluorescence.

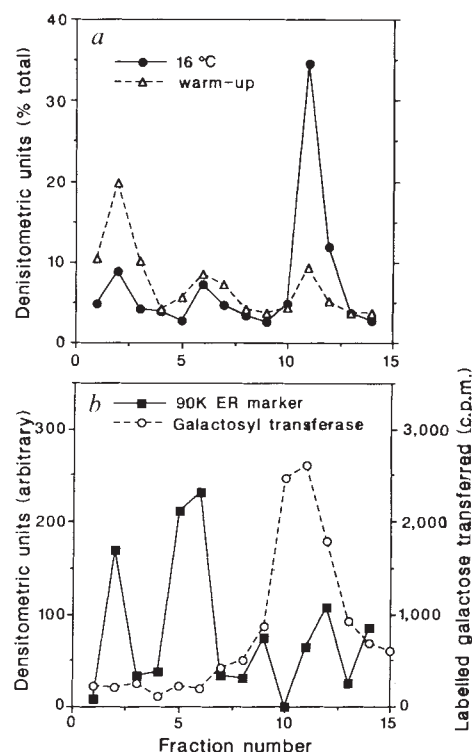


FIG. 2 Subcellular fractionation of class I molecules. With temperature changes, class I molecules (a) redistribute from mainly heavy (ER-like) to mainly light (Golgi-like) fractions, whereas the ER (assessed with a protein of relative molecular mass 91,000 (91K) recognized by the anti-ER antiserum) and the Golgi (galactosyl transferase activity) marker remained unchanged (b). Total intensity in each condition is roughly equal.

METHODS. Subcellular fractionation was performed as previously described⁸. Cells (6×10^7) were metabolically labelled with [³⁵S]methionine at 37 °C for 3 h and then chased in unlabelled medium and incubated either at 37 °C for 2 h, 16 °C for 2 h and then 37 °C for 5 min, or at 16 °C for 2 h and then 37 °C for 30 min. After washing and homogenization, the postnuclear supernatant was centrifuged (40,000 r.p.m. for 2 h in a Beckman SW41 rotor) through a discontinuous sucrose gradient, and 16 fractions each containing 20 drops were collected from the bottom of the tubes. For class I molecules and ER marker, each membrane fraction was solubilized in 1% Triton X-100 and immunoprecipitated with antibodies as described in Fig. 1 legend, run on 10% SDS/PAGE, and then exposed for autoradiography. Specific bands were scanned by a densitometer (Ultrascan XL, LKB Instruments). For galactosyl transferase activity, each membrane fraction was incubated for 1 h at 37 °C in 5×10^4 c.p.m. UDP-galactose-4,5-³H(N) (NEN-DuPont), 0.28 mg ovalbumin (grade V, Sigma), 50 mM Tris buffer, pH 7.5, 20 mM MnCl₂, and 0.5% Triton X-100 in a total volume of 40 μl . Samples (30 μl) were spotted on Whatman 3-MM filter paper, dried, precipitated with trichloroacetic acid, washed, dried and then counted.

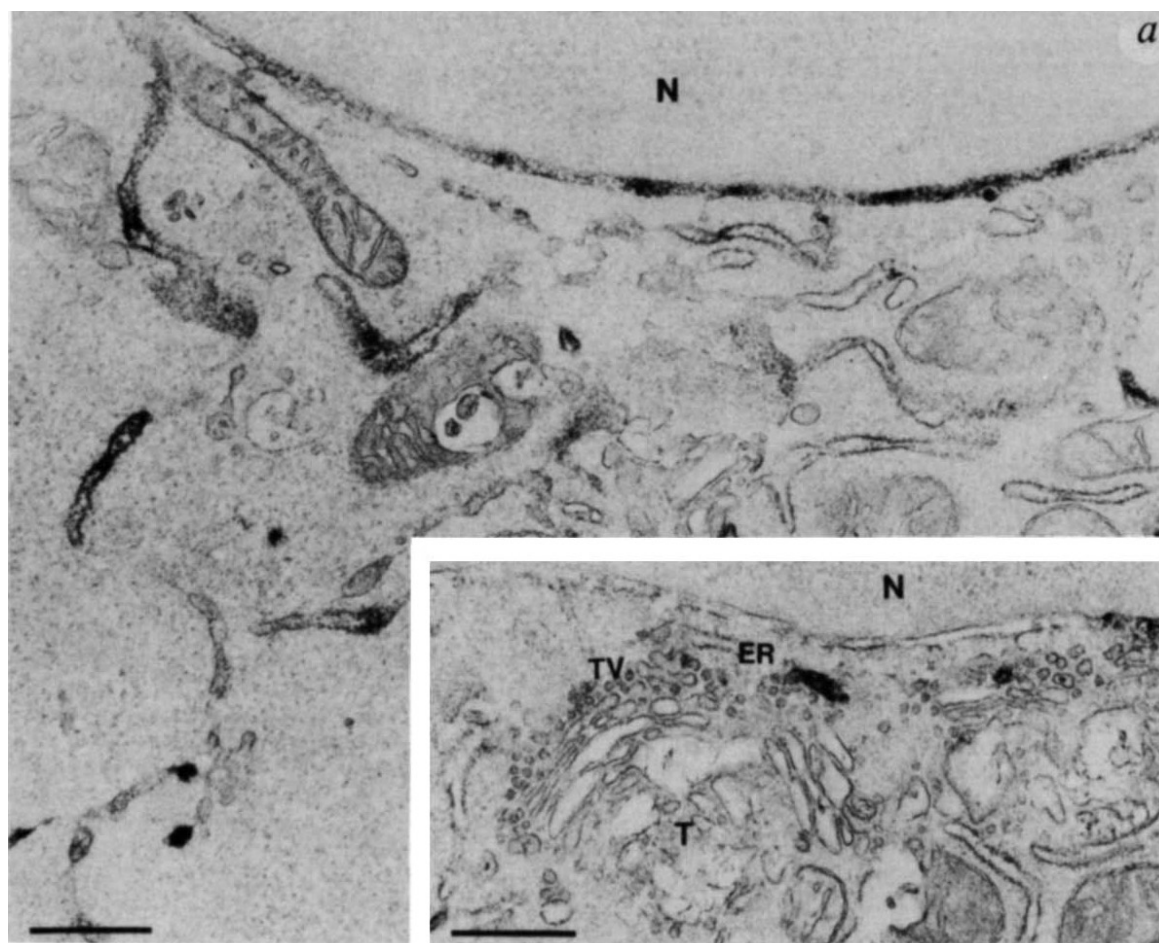
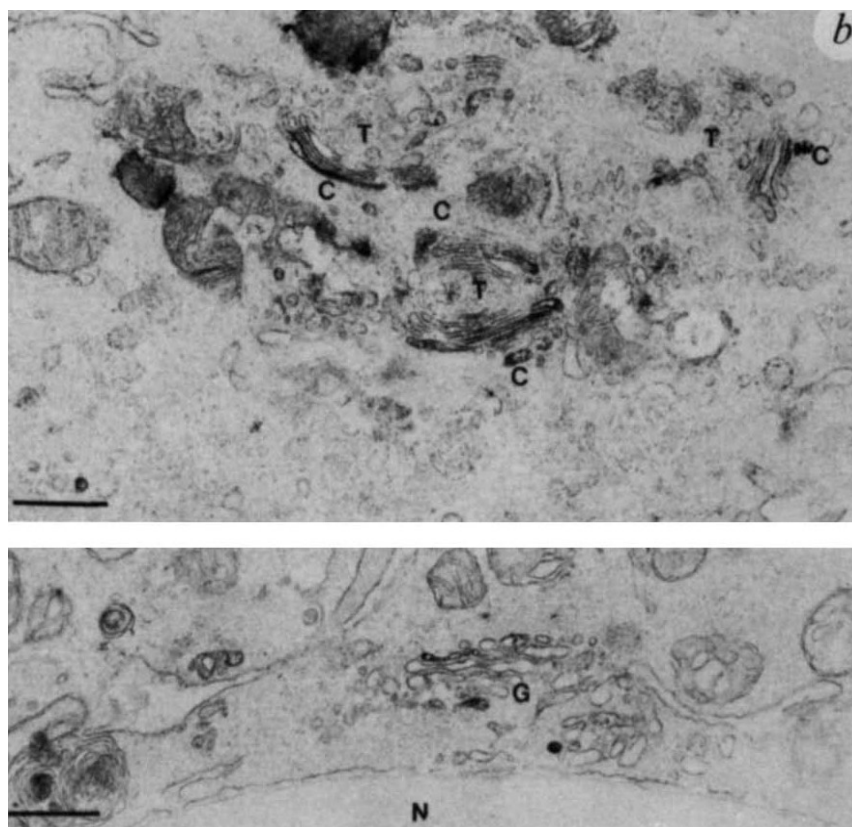


FIG. 3 Temperature-induced shift of class I distribution examined by immunoperoxidase electron microscopy. *a*, At 37 °C, the dark peroxidase reaction product, indicating the distribution of class I, appeared in the nuclear envelope and tubulo-vesicular elements of the ER (including the rough ER). In these structures, the staining appeared to be concentrated in discrete patches. The Golgi apparatus, shown in the inset, did not stain for class I molecules. *b*, At 16 °C, the reaction product was localized mainly to the Golgi apparatus, usually within the *cis*-most two cisternae. The ER cisternae and the nuclear envelope (bottom panel) were not stained. Scale bar, 0.5 μ m; N, nucleus; ER, endoplasmic reticulum; TV, transition vesicles; T, *trans* face of the Golgi apparatus.

METHODS. Immunoperoxidase electron microscopy was performed as previously described⁴. Cells on coverslips were incubated under appropriate temperatures, fixed with paraformaldehyde containing lysine and periodate, permeabilized with saponin, and labelled with anti-class I antibody (SF1-1.1.1) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Cooper Bio-medical). After reaction with diaminobenzidine hydrochloride and hydrogen peroxide, cells were prepared for electron microscopy.



rewarming the cells to 37 °C, the same population of class I molecules redistributed mainly to the ER region (Fig. 2a). These results are consistent with the morphological observations, although they do not precisely define the structures involved.

We therefore examined the distribution of class I molecules by immunoperoxidase electron microscopy. At 37 °C (Fig. 3a) the molecules were distributed throughout the ER, including the nuclear envelope, but were mainly located in smooth regions of the ER. No staining was observed in Golgi cisternae. Temperature-induced redistribution (Fig. 3b) resulted in a loss of ER staining. Instead, the Golgi cisternae became stained, most of the staining being confined to what seemed to be the *cis*-most one, two, or occasionally three cisternae.

Our study indicates that class I molecules that do not migrate along the secretory pathway but instead seem to be retained in the ER can in fact cycle through a Golgi-like structure. Nocodazole treatment indicates that this cycling can occur at 37 °C. Much evidence points to the existence of a post-ER recycling pathway for the retention in the ER of some proteins that reach the pre-Golgi compartments of the secretory pathway⁹, although the structures responsible remain unknown. Could they be part of the Golgi apparatus? Indeed, the human KDEL receptor proposed to retrieve ER luminal proteins that possess the C-terminal signal KDEL (Lys-Asp-Glu-Leu), is localized to a Golgi-like structure¹⁰. Despite this, at least one ER resident protein, the KDEL-containing glycoprotein ERP99, presumably retained through KDEL receptor-mediated retrieval, does not undergo Golgi-associated carbohydrate processing¹¹. This observation is consistent with our findings for the class I pathway. Movement of glycoproteins into the Golgi apparatus is indicated by characteristic changes in the structure of *N*-linked carbohydrate sidechains¹². However, the sidechains of class I molecules did not develop the sensitivity to endo D or resistance to endo H that is associated with these changes. It is not clear whether the lack of processing is because class I proteins do not reach the region of the Golgi apparatus responsible for such modifications or whether proteins earmarked for recycling are not substrates for Golgi processing.

We suggest on the basis of the morphological data that the recycling proteins accumulate in a structure that includes the *cis*-most cisternae of the Golgi apparatus, and that this may be a subcompartment for the sorting of components between an anterograde and a retrograde pathway. Exactly how many cisternae of a stack make up this remains unknown. Subcompartmentalization at the *cis* face of the Golgi is reminiscent of the complex network of vesicles at the *trans* face called the *trans*-Golgi network¹³. We therefore propose that the recycling compartment should be called the '*cis*-Golgi network'. Our studies thus imply additional complexity to the quality-control mechanisms available to the secretory pathway. □

Transcription by single molecules of RNA polymerase observed by light microscopy

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THE kinetics of transcription by *Escherichia coli* RNA polymerase relate directly to the regulation of transcription and to the properties of processive enzymes in general¹, but analysis of RNA polymerase movement along the DNA template has so far been limited to the study of populations of enzyme molecules. The ability to view nanometre-sized particles with the light microscope^{2,3} suggested a method of monitoring transcription by individual RNA polymerase molecules. We describe here the behaviour of 40-nm-diameter particles of colloidal gold attached to the ends of DNA molecules being transcribed by RNA polymerase immobilized on a glass surface. The tethered gold particles are released from the surface at times after addition of nucleoside triphosphates that are consistent with the kinetics of transcription by RNA polymerase in solution. Analysis of the brownian motion of the gold particles enabled us to measure the movement along the template DNA of individual polymerase molecules.

First, we determined the activity of *E. coli* RNA polymerase that had been immobilized on glass. We used a DNA template (template 1; Fig. 1a) that contains the very strong A1 promoter of phage T7 preceding a portion of the *E. coli trp* leader and the *rrnB* T1 terminator⁴. RNA polymerase molecules that initiate transcription of this template in the presence of ApU dinucleotide, ATP, CTP and GTP stall before addition of U-21 and after addition of A-20 (ref. 5). When UTP is added, RNA polymerase resumes transcription and either terminates at the *rrnB* T1 terminator or falls off the end of the DNA. We purified stalled 'A20 complexes' prepared with ³²P-labelled DNA template 1 (Fig. 1a)⁶ and incubated a sample containing about 3.5×10^9 A20 complexes on a borosilicate glass coverslip. After extensive washing (see legend to Fig. 1), 10–20% of the complexes remained bound to the glass. After addition of nucleoside triphosphates (NTP), 50–70% of these immobilized complexes resumed transcription with an elongation rate and a termination efficiency at the *rrnB* T1 terminator that were indistinguishable from those of A20 complexes in solution (data not shown).

We next prepared immobilized transcription complexes with a 40-nm-diameter colloidal gold particle attached to the end of DNA template 2 upstream from the RNA polymerase. We immobilized transcription complexes containing upstream-biotinated template 2 (Fig. 1a) on glass and then tagged the biotinated ends with streptavidin-coated gold particles (Fig. 1b). When we observed these specimens with video-enhanced differential interference contrast microscopy, three classes of gold particles were distinguishable. Particles in one class were immobile and tightly attached to the coverslip. Members of another class exhibited rapid brownian motion, but each remained in a region $<1 \mu\text{m}$ from its initial location on the coverslip surface even after >10 min. This behaviour would be expected for particles attached by a flexible tether to a fixed point on the surface. These 'tethered' particles were distinguished from the third class of particles, which also exhibited

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brownian motion but diffused rapidly in and out of the field of view. This is the behaviour observed for 40-nm gold particles free in solution (Stoke's law diffusion coefficient, $D = 1.1 \times 10^7 \text{ nm}^2 \text{ s}^{-1}$), which have a mean residence time in a $1\text{-}\mu\text{m}$ diameter volume of only 0.022 s.

The numbers of immobile and tethered gold particles were roughly proportional to the numbers of A20 complexes applied to the slide (over the range of 10^6 – 10^9 complexes). When we examined particles tethered to transcription complexes containing templates 4 or 5 biotinized at the downstream end, there were much greater ranges of brownian motion. Tethered particles were not detected on control slides prepared with A20 complexes containing nonbiotinized DNA templates, or with biotinized DNAs alone at 25-fold higher concentration. We concluded that the tethered particles were attached to the ends of RNA polymerase-bound DNA templates.

When we initiated transcription by the gold-labelled, immobilized transcription complexes by addition of NTP to a concentration of 1 mM each, we observed two changes in the behaviour of the tethered particles. For a small fraction of the tethered particles, the range of brownian motion increased substantially,

as would be expected from a lengthening of the DNA tether caused by transcription. Occasionally such particles released from the coverslip surface after a variable time delay. Other particles released without noticeable change in the range of brownian motion. In >80 experiments using four different templates (templates 2–5; Fig. 1a), out of 180 tethered particles watched for an average of 112 s before the addition of NTP, only two released from the glass surface, whereas 59 of the remaining 178 particles released an average of 128 s after NTP was added (Table 1).

In these preliminary experiments, most of the changes in the range of tethered-particle brownian motion after NTP addition were sudden. Because the streptavidin-coated gold particles contain multiple biotin-binding sites, a particle can attach to multiple DNA tethers and can show a sudden change in the range of brownian motion when one of them is released from a transcription complex. This could also explain why most tethered particles did not release from the glass, as only 50–70% of immobilized complexes were transcriptionally active.

To minimize the problem of multiple attachments, we decreased the surface density of transcription complexes to

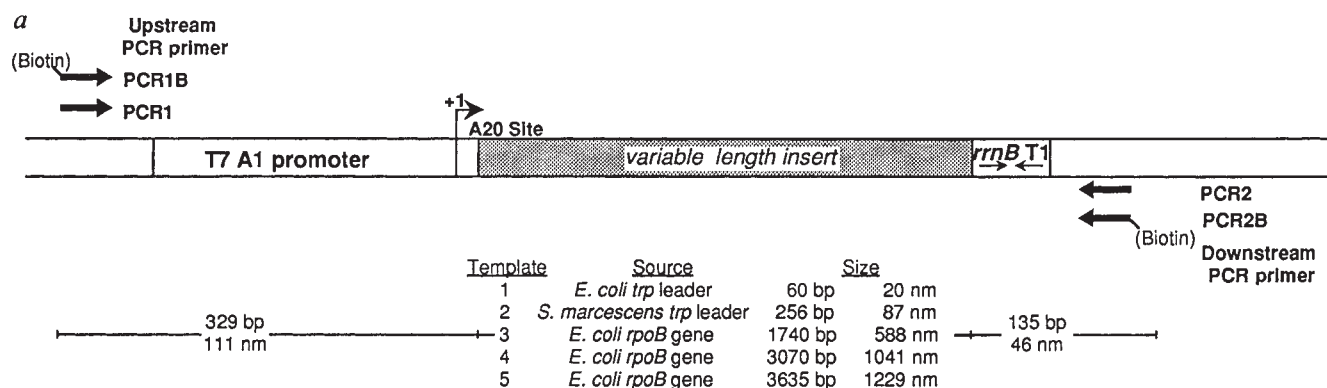
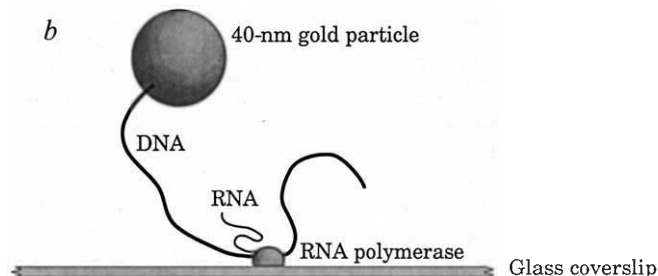


FIG. 1 a, Schematic representation of the DNA molecules used as transcription templates. The lengths of segments of DNA were calculated assuming that 10 bp correspond to 3.38 nm (ref. 9). b, Illustration of the immobilized transcription complex labelled with a streptavidin-coated, 40-nm-diameter gold particle at the upstream end of the biotinized DNA template.

METHODS. a, DNA templates were prepared by 30 cycles of the polymerase chain reaction (PCR) from ~100 ng plasmid DNA (refs 4, 10). To place biotin upstream from the A20 site, the normal upstream PCR primer (PCR1) was replaced with a primer (PCR1B) of identical sequence that contained biotin covalently attached to the 5' OH group through a 6-carbon spacer and phosphate linkage (biotinized primers were prepared by Midland Certified Reagent, Texas, using the Biotin dX reagent). To prepare transcription templates with biotin downstream from the *rrnB* terminator, the downstream PCR primer (PCR2) was replaced with a biotinized primer (PCR2B). Template 1 was prepared from plasmid pRL458 (ref. 4), which contains the T7 A1 promoter, a 60-bp insert, and the *rrnB* terminator region. Template 2 was prepared from a similar plasmid that contained a portion of the *Serratia marcescens trp* leader inserted in reverse orientation between the A20 site and the *rrnB* terminator segments. During conventional *in vitro* transcription experiments, no premature termination was observed in this fragment (data not shown). To create DNA templates 3–5 that could support transcription of >1,000 bp (~300 nm), we inserted segments of one of the largest *E. coli* genes, *rpoB*, which encodes the second largest subunit of RNA polymerase. Because *rpoB* must be completely transcribed *in vivo*, these fragments are unlikely to contain natural transcription terminators. b, Streptavidin-coated colloidal gold was prepared by suspending 40-nm-diameter colloidal gold particles (E-Y Labs) in an equal volume of 10 mM Tris-HCl, 1 mM Na₂EDTA, pH 7.4 (TE buffer), containing 8 mg ml⁻¹ BSA and 2 mg ml⁻¹ biotinamidocaproyl-BSA (Sigma). After incubation on ice for 15–60 min, the protein-coated gold particles were collected by centrifugation for 15 min at 6,000 r.p.m. in a Sorval HS-4 swinging bucket rotor at 4 °C. The gold particles were resuspended in TE buffer containing 1 mg ml⁻¹ streptavidin and incubated on ice for 15–30 min. The gold particles were collected as described above, resuspended in the original volume in PTC buffer (20 mM Tris-acetate,



pH 7.9, 125 mM KCl, 4 mM MgCl₂, 0.1 mM Na₂EDTA, 0.1 mM dithiothreitol, 4% glycerol, and 20 μg ml⁻¹ acetylated BSA) supplemented with 1 mg ml⁻¹ BSA and stored on ice; streptavidin-coated gold was prepared freshly each day. A20 transcription complexes with a biotinized DNA template were prepared as described⁶. To assemble the gold-labelled immobilized transcription complexes, 10 μl transcription complexes was placed between two lines of silicone vacuum grease in the centre of a borosilicate glass coverslip (Clay-Adams No. 3223) in a humidified chamber and incubated for 15 min at 20 °C. To remove unbound complexes, the solution over the coverslip was partially replaced 10 times by the simultaneous withdrawal of 10 μl solution and addition of 10 μl PTC buffer, and BSA was then added to a concentration of 1 mg ml⁻¹. After incubation for 5 min, 5 μl of the suspension of streptavidin-coated gold particles was added. After 5 min incubation, the solution was partially replaced 3 times with 10 μl PTC buffer supplemented with 1 mg ml⁻¹ BSA and 80 μg ml⁻¹ heparin. In some experiments, biotin was added to 100 μg ml⁻¹ and the solution covered with a second glass coverslip to form a flow chamber. To initiate transcription, 50 μl PTC buffer supplemented with 1 mg ml⁻¹ BSA, 80 μg ml⁻¹ heparin and 1 mM each of ATP, GTP, CTP and UTP was allowed to flow into the chamber.

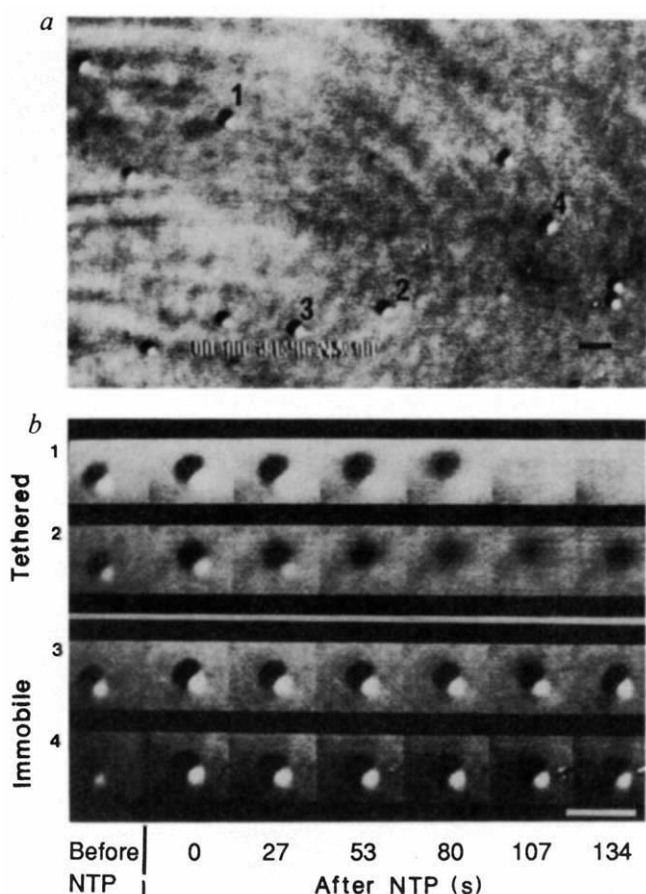


FIG. 2 Video-enhanced differential interference contrast light-microscope images of immobilized transcription complexes labelled with colloidal gold particles. The specimen was prepared as described in Fig. 1, using template 3 biotinized at the upstream end (Fig. 1a). To accentuate the blurred appearance of particles undergoing brownian motion, 2.1 s of video (64 frames) was averaged to produce each image. Scale bars, $1\ \mu\text{m}$. **a**, A microscope field containing tethered and immobile gold particles before the addition of NTP. Particles 1 and 2 showed limited brownian motion consistent with their being tethered by ~ 300 bp of DNA. Particles 3 and 4 were immobile, consistent with direct attachment to the coverslip surface. **b**, Changes in particle brownian motion during transcriptional elongation. The first column shows magnified images of particles 1–4 taken from **a**. Subsequent columns show the images of the same particles taken immediately (0 s) and at 27, 53, 80, 107 and 134 s after transcription was initiated by the addition of NTP. Images of particles 1 and 2 become increasingly blurred after NTP addition because transcriptional elongation lengthens the DNA tether linking the particle to the coverslip, resulting in increased range of particle brownian motion. Particles 1 and 2 were released from the surface at 87 and 135 s after NTP addition, respectively. Particles 3 and 4 showed no brownian motion and did not release from the surface.

METHODS. Differential interference contrast microscopy was done as previously described¹¹. With differential interference contrast optics, the image of a single colloidal gold particle consists of adjacent white and black ovoids on a grey background. Focus on immobile gold particles bound to the coverslip surface was maintained by manual adjustments. Video was recorded with a Newvicon video camera (interlaced scans, 30 Hz frame rate, 3 MHz bandwidth) and an S-VHS video recorder. Some sequences were dubbed to an optical memory disk recorder (Panasonic) before analysis. Recorded sequences were transferred using a full-frame digital time-base corrector to a 512 pixel per line digital image processor (1 pixel ≈ 40 nm) which averaged non-overlapping, 64 frame segments of the input video. Averages were stored in a real-time digital image storage device (Applied Memory Technologies, California). Optical mottle was removed by subtracting a background image¹².

$0.6\ \mu\text{m}^{-2}$ so that only 15% of the complexes were close enough to a neighbouring complex to allow a single particle to attach to both (assuming that the complexes bind independently to the glass and that the tether length is 147 nm for upstream DNA, streptavidin–biotin linkage, and gold particle radius.) Dilution of the complexes greatly reduced the number of tethered particles visible; at the dilutions required for complexes with longer initial tethers (for example those with downstream-labelled templates 3–5; Fig. 1a), there were not enough tethered particles for experiment. To ensure that new DNA–particle attachments did not form during elongation, we saturated residual streptavidin sites with biotin before NTP addition.

Using these conditions, there were many more particles whose ranges of brownian motion increased smoothly after addition of NTP. Frequently, such particles subsequently detached from the surface. There were still many immobile particles, and some

tethered particles did not detach from the surface within 5 min of NTP addition. We attribute this to nonspecific binding of some particles to the surface or to inactivation of some of the immobilized complexes. Nevertheless, our observations are consistent with the view that the characteristic motion of the tethered gold particles was a result of their attachment to the template DNA in immobilized transcription complexes, and that increased brownian motion and release of these particles after addition of NTP were both consequences of transcription.

To analyse the changes in tethered-particle brownian motion caused by transcriptional elongation on templates 3–5, we averaged video frames from successive 2.1-s intervals after NTP addition (Fig. 2). During each 2.1-s interval, the rapidly moving particles should assume positions distributed throughout the entire volume in which they are constrained by the DNA tether. Thus, the interval-averaged frames contain a blurred image of

TABLE 1 Release of gold particles tethered on the upstream end of DNA transcription templates

DNA template*	Total particles observed	Before addition of NTP		After addition of NTP		Transcription length (nm)§
		Number released	Mean time observed (s)	Number released	Time to release (s)† (mean $\pm$ s.d.)	
2	21	0	102	10	81 ± 41	109
3	42	1	169	7	140 ± 98	633
4	29	0	88	10	165 ± 45	1,074
5	88	1	96	32	128 ± 102	1,267

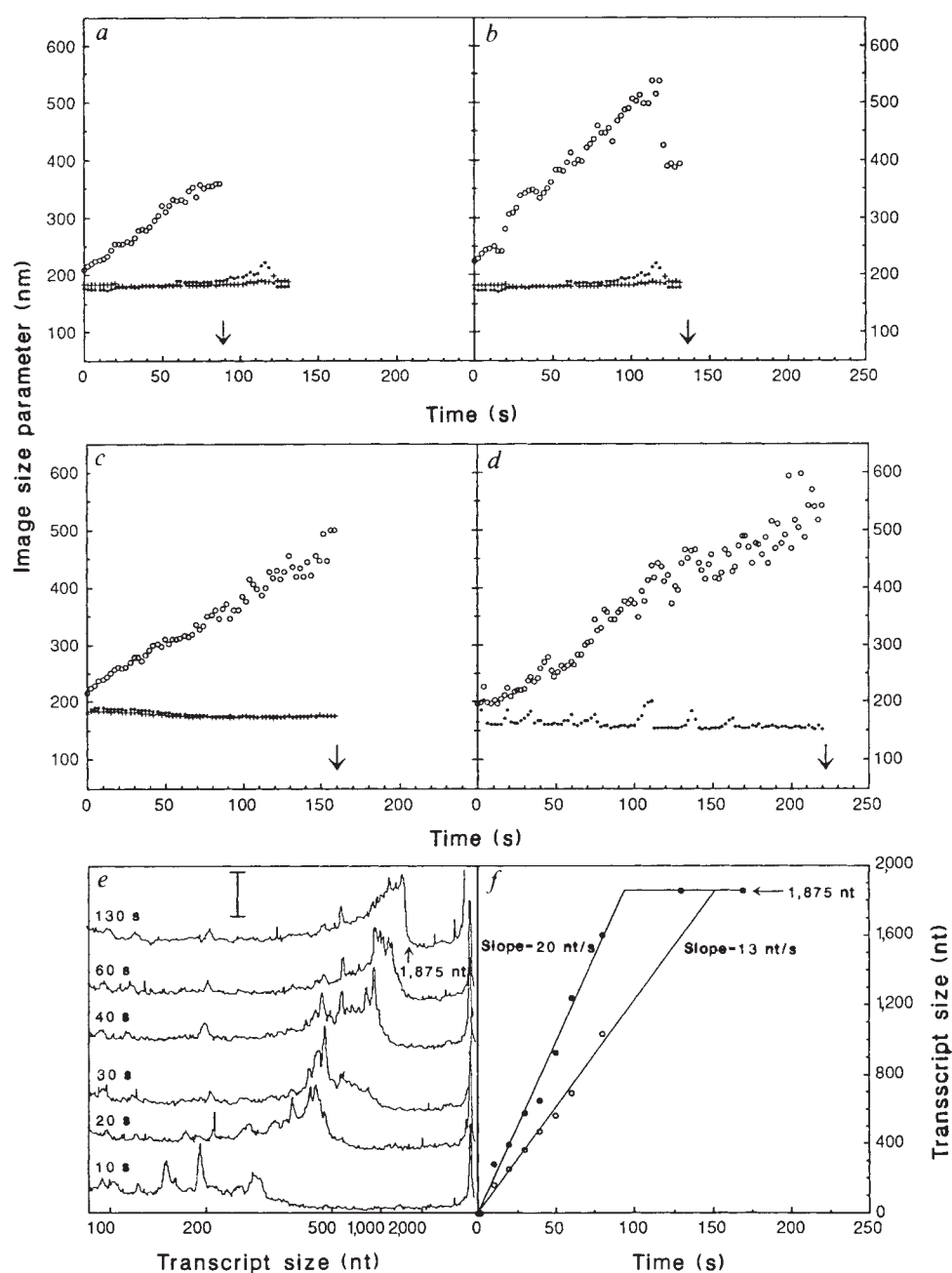
* See Fig. 1a.

† Data are compiled from experiments in which the surface density of immobilized transcription complexes ranged from 0.3 to $15\ \mu\text{m}^{-2}$. Most data for templates 2 and 3 were obtained at high surface densities without adding free biotin before addition of NTP, whereas those for templates 4 and 5 were obtained at low surface densities and with the addition of biotin. In all cases, some particles were released before the time predicted for completion of transcription at $13\text{--}20\ \text{nt s}^{-1}$ (Fig. 3f), presumably because particles were released when some complexes terminated transcription prematurely.

§ Distance from the A20 position to the known position of transcription termination at the *rrnB* T1 termination site on the template. Calculated assuming 10 base pairs correspond to $3.38\ \text{nm}$ (ref. 9).

FIG. 3 Elongation and termination of single immobilized transcription complexes compared with population-averaged elongation and termination kinetics of transcription complexes in solution. *a-d*, Gold particle-labelled immobilized transcription complexes. Plots show the size ('image size parameter') of particle images in interval-averaged video frames as a function of time after NTP addition for specimens prepared and observed as in Fig. 2. The image size for tethered gold particles ($\circ$) reflects the range of particle brownian motion. Each tethered particle detached from the surface at the time indicated by the arrow. Data taken from the same video recordings for reference particles that were immobile on the surface ($\bullet$, $+$) demonstrate that slow changes in image size are not due to drift in the microscope focus. Tethered particles in *a* and *b* were taken from the same field (Fig. 2, particles 1 and 2, respectively) and the immobile particle data in the panels are identical (Fig. 2, particles 3 and 4). DNA templates (see Fig. 1*a*); *a* and *b*, 3; *c*, 4; *d*, 5. *e*, *f*, Transcription complexes in solution. Elongation of A20 complexes prepared with template 3 was initiated with NTP at 1 mM each plus [α - 32 P]GTP and stopped after the indicated times by addition of an equal volume of 10 mM urea, 200 mM Tris-borate, pH 8.3, 5 mM Na₂ EDTA. *e*, Densitometer tracings of an autoradiogram obtained after electrophoretic separation of the RNA transcripts. Scale bar, 0.4 absorbance units. *f*, Estimate of maximum ($\bullet$) and average ($\circ$) transcript length versus time for the experiment in *e*. Several data not shown in *e* are included. Estimates of the average transcript length after 60 s were not possible because of the limited electrophoretic resolution of RNA > 500 nucleotides long.

METHODS. All reactions conducted at 20 °C in PTC buffer + 1 mM NTP. *a-d*, Each datum was calculated from the average of 64 video frames obtained as in Fig. 2. The image size parameter includes both the intrinsic size of the particle image (as seen with immobile particles) and the blurring of the particle image caused by three-dimensional brownian motion during the averaging interval. The size parameter represents the characteristic size of the image (that is, the radius at which intensity falls off by a factor of $1/e$) measured in the direction normal to the differential interference contrast shear axis. The size parameter was determined by fitting an empirical parameterized image shape function to the averaged images using a two-dimensional non-linear modelling routine (written by B. Grossman) based on the implementation of the Levenberg-Marquardt algorithm in program MRQMIN (ref. 13). The shape function was of the form $f(x, y) = e^{-(x+v)^2 - y^2} - e^{-(x-v)^2 - y^2}$, where v is the offset parameter (described below). In addition to the image size parameter already described, adjustable parameters modified the shape function for changes



in the image position, in the microscope illumination intensity and in the image contrast. Three additional parameters (the offset parameter v , the rotation angle necessary to make the shape function x axis fall along the differential interference contrast shear axis, and the ratio of image sizes along and normal to the shear axis) reflect properties of the microscope optical system; each of these parameters was fixed at a predetermined value throughout the experiments. *e* and *f*, A20 complexes containing DNA template 3 were prepared as described⁶. Samples were removed at the indicated times after addition of NTP to 1 mM each and electrophoresed on a 4% polyacrylamide, 7 M urea gel⁴. Kodak XAR-5 film was exposed directly to the gel and then analysed using a Molecular Dynamics model 300 computing densitometer.

the particle, the size of which increases as the range of brownian motion increases. The progressive increase in tethered-particle image size after NTP addition (Fig. 2*b*, particles 1 and 2) is consistent with progressive elongation of the DNA tether due to transcription. By contrast, the images of immobile particles in the same video field (Fig. 2*b*, particles 3 and 4) did not change.

To quantitate the changes in tethered particle image size, we

fitted an empirical shape function to the interval-averaged particle images using digital image processing techniques (Fig. 3), giving an 'image size parameter' that is proportional to the diameter of the blurred image. Simulations with artificial images suggested that an increase in the image size parameter of 1 nm corresponds to an increase in tether length of about 1.3 nm over most of the range of the experimental data (values for the tether

length given by $1.3\alpha + 64$ nm, where α is the image size parameter, differ by no more than 5% from the calculated values for $255 \leq \alpha \leq 646$ nm).

In experiments on templates 3–5, there was a smooth increase in the image size parameter (Fig. 3a–d), confirming that the increase in the range of brownian motion was due to continuous elongation of the tether and not to sequential severing of discrete attachments between the particle and the coverslip. Occasionally, we observed particles for which there was a sudden large change in the image size parameter (for example, see Fig. 3b after 123 s) or for which the mean particle position shifted 0.5 μ m or more. We attribute these behaviours to detachment of a DNA tether in the small fraction of the particles expected to be linked by more than one tether, or to transient nonspecific interactions between the DNA and particle, DNA and surface, or particle and surface.

Measured elongation rates of individual immobilized transcription complexes are consistent with elongation rates obtained using conventional assays for transcription complexes in solution. For experiments at low surface densities of complexes containing templates 3–5, the initial rate at which the size parameter increased ranged from 1.6–3.7 nm s⁻¹ (mean 2.3 nm s⁻¹; $n = 5$). These rates correspond to tether length changes of 2.1–4.8 nm s⁻¹ or 6.2–14 nucleotides per second (nt s⁻¹). For comparison, we measured transcription of template 3 by RNA polymerase in solution under conditions identical to those used for the immobilized specimens. Depending on the method of estimation, RNA polymerase in solution transcribed at 13–20 nt s⁻¹ (Fig. 3e, f). For the immobilized enzyme, the time between addition of NTP and release of the particle provides another measure of elongation rate, assuming that release coincides with arrival of polymerase at the *rrnB* terminator. The corresponding rates would be 17 nt s⁻¹ for template 3 (average of data in Fig. 3a, b), 20 nt s⁻¹ for template 4 (Fig. 3c), and 17 nt s⁻¹ for template 5 (Fig. 3d), which is in good agreement with the elongation rate for RNA polymerase in solution (Fig. 3e, f). Our data are as yet insufficient to answer two important questions about transcription: (1) do different RNA polymerase molecules in solution transcribe at different intrinsic elongation rates, as the differences we observed for immobilized complexes suggest, and (2) does RNA polymerase translocate on the DNA in fundamental steps larger than one base-pair^{7,8}.

We conclude that individual RNA polymerase molecules stop no longer at any site than would be predicted for normal transcriptional pausing^{1,4–6} and transcribe at an intrinsic rate of elongation that does not vary substantially with time. They do not stop at random positions for long periods, a feature that would have been undetectable in previous experiments, all of which averaged properties of large populations of enzyme molecules. Further use of this technique to study single molecules of processive enzymes may reveal additional features that were not evident from previous studies. □

Accurate prediction of the stability and activity effects of site-directed mutagenesis on a protein core

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THEORETICAL prediction of the structure, stability and activity of proteins, an important unsolved problem in molecular biology, would be of use for guiding site-directed mutagenesis and other protein-engineering techniques. X-ray diffraction studies have provided extensive structural information for many proteins, challenging theorists to develop reliable techniques able to use such knowledge as a base for prediction of mutants' characteristics. Here we report theoretical calculation of stabilization energies for 78 triple-site sequence variants of λ repressor characterized experimentally by Lim and Sauer¹. The calculated energies correlate with the mutants' measured activities; active and inactive mutations are discriminated with 92% reliability. They correlate even more directly with the mutants' thermostabilities, correctly identifying two of the mutants to be more stable than the wild type.

Free-energy stability calculations for several single-site mutations have been reported, including Leu 33 $\rightarrow$ Ala in trypsin², Thr 157 $\rightarrow$ Val (ref. 3) and Arg 96 $\rightarrow$ His (ref. 4) in T4 lysozyme. These calculations require hundreds of hours of supercomputer time for even a simple single-site mutation. This makes it impractical to test the method on enough cases to establish statistical significance of experimental correlations, and calculations on

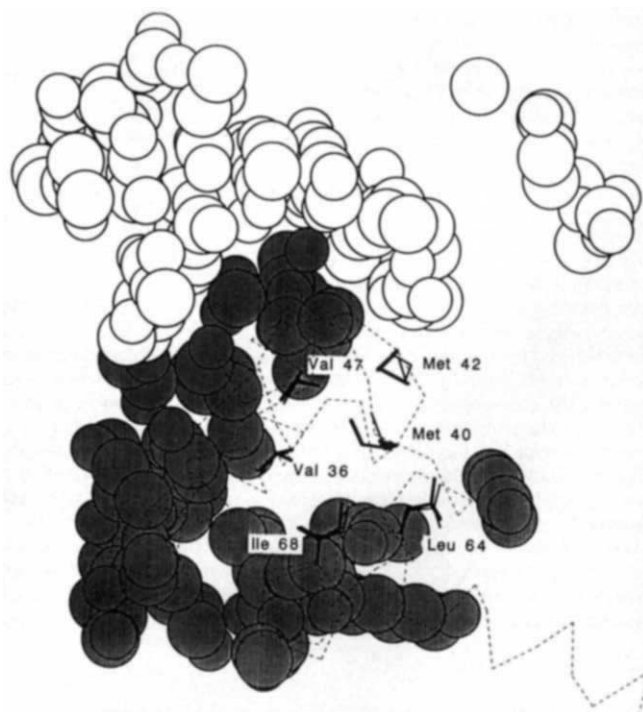


FIG. 1 The 'molten zone' of side-chains allowed to move in the prediction is shown as solid lines (predicted wild-type structure (thin lines), versus the X-ray structure (thick)), in the context of the rest of the protein, shown as van der Waals surfaces (shaded circles), and the bound DNA (light circles), in a 4 Å slice passing through the protein core. The C α -trace of the main-chain is shown as a dashed line. Figure made using MacIcmdad.

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multiatom substitutions (for example Val → Met), core mutations and multisite mutations are problematic^{5,6}.

There is a need for methods that are simple and fast enough to be tested against a very large set of mutations, and which do not require adjustment of their parameters to fit each mutant. Recently, we reported a method for prediction of the conformation of side-chains in protein cores that is rapid, insensitive to starting conformation, uses only effective atomic radii as parameters (see Table 1) and provides accurate structure predictions even for entire protein cores⁷. We have adapted such methods to prediction of mutant structure and energetics. In doing so, several simplifying assumptions were made: (1) the mutant structure should mostly resemble the wild-type, except for side-chain shifts in a zone around the mutations. Specifically, side-chains in this 'molten zone' are moved through their full torsional range, seeking the conformation that best packs them together, while the surrounding side-chains and all main-chain are held fixed. (2) To choose the best structure, the method uses an energy function (E_{calc}) including only van der Waals interactions and a simple torsional potential; bond length and angle deformations, electrostatics and solvation effects are ignored. (3) Energetic analysis of the predicted mutant structures uses simple averaging over the lowest energy conformations generated during annealing, focusing entirely on the folded state of the protein; no attempt is made to calculate a true free energy using molecular dynamics, simulation of the unfolded state, and perturbation theory. Instead, the method searches the space of possible conformations for well-packed structures; its calculated energy for each mutant (E_{calc}) is simply the average of the packing energy for the 1,000 best conformations found.

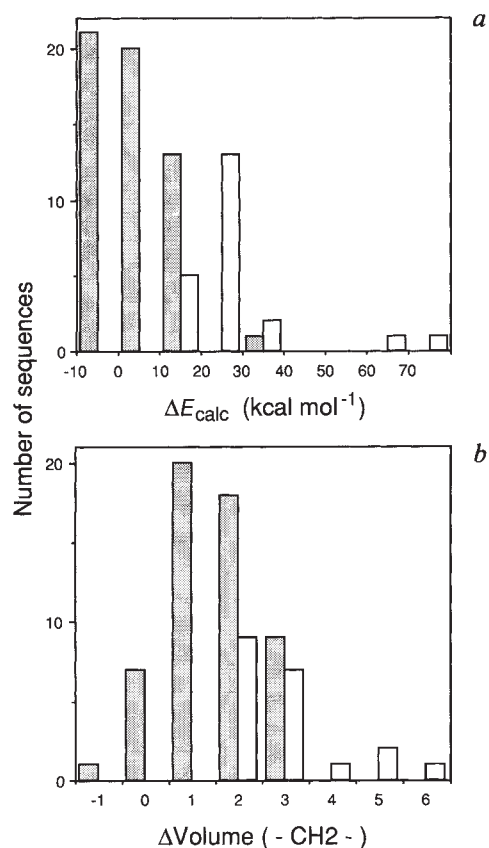


FIG. 2 Discrimination of active versus inactive mutations. *a*, Histogram showing the distribution of active (grades 1–5 at 26 °C) and inactive mutants (grade 0) over the range of calculated energies (relative to wild-type). Hatched bars, active; open bars, inactive. *b*, Equivalent histogram showing the distribution of active versus inactive mutants over the range of their volumes (relative to wild-type, in units of methylene groups; reproduced from ref. 1).

Some of these assumptions could be wrong; fortunately experiments have recently been done which can test the method's predictions^{1,8}. Using oligonucleotide synthesis techniques to permute simultaneously three different sequence positions in λ repressor (residues 36, 40 and 47) through five different hydrophobic amino acids (Ile, Leu, Met, Phe and Val), Lim and Sauer isolated clones of 78 of the $5 \times 5 \times 5 = 125$ possible mutants, and

TABLE 1 Energetic analysis of predicted structures

Mutant	E_{calc}	Activity grade*	T_m (°C)*	Mutant	E_{calc}	Activity grade*	T_m (°C)*
VMV (wt)	-35.63	5	56	LIM	-34.93	2	
VIV	-33.68	5		LLL	-34.26	2	
IMV	-37.13	5	59	VFI	-16.09	2	
VVI	-31.77	5		IFV	-18.61	3	
VMI	-37.77	5		MII	-37.66	4	
VLI	-36.91	5		MIM	-32.99	2	
VII	-36.59	5		LFV	-17.22	2	
IVI	-33.88	5	54	IFI	-19.77	3	
III	-38.88	5		FII	-18.87	2	
ILI	-39.39	5		LFI	-18.67	2	
LVV	-29.53	2		FMI	-19.98	1	
VML	-35.18	2		MFI	-17.37	2	
VLL	-34.91	2		FLI	-19.30	2	
LMV	-36.09	2		LFM	-18.52	1	
LLV	-34.86	2		FML	-19.38	1	
LIV	-34.84	3		IFL	-17.50	2	
IVL	-32.02	2	52	FFI	+0.11	1	47
LLI	-37.50	2	60	VVF	-6.75	0	
MLI	-38.21	2		FVV	-11.60	0	
MMI	-34.77	2		FLV	-15.09	0	52
VMI	-37.94	2		FVM	-14.89	0	
VVV	-30.72	4		VLV	-9.95	0	
IVV	-30.93	4		LVF	-10.43	0	
VVL	-30.24	2	51	VIF	-9.05	0	
VMV	-32.10	2		IVF	-6.93	0	
VVM	-32.37	2		MVF	-10.35	0	
MMV	-34.47	2		VFM	-17.26	0	
IVM	-35.36	2		FVL	-13.91	0	
LVI	-34.02	3	54	LLF	-13.39	0	
VLM	-35.79	2		FIM	-18.52	0	
VFV	-15.94	2		FLM	-18.01	0	
VMM	-36.33	2		IMF	-10.87	0	
LVM	-34.81	2		FMM	-18.95	0	
VIM	-35.49	2		MLF	-13.25	0	
ILM	-37.07	2		LIF	-12.16	0	
IMM	-38.82	2		FVF	+26.27	0	
FVI	-15.45	2		FFM	+0.59	0	
LII	-37.75	4		LFF	+3.07	0	
LLM	-37.67	2		FFF	+41.47	0	

Predicted energetics (in kcal mol⁻¹) for the 78 repressor mutants, each designated by single-letter amino-acid codes of the three mutated residues (36, 40, 47). Lim and Sauer's experimental measurements of repressor activity are reprinted next to each mutant, along with the melting temperatures of the nine mutants they have purified and measured¹. A modified version of the published method⁷ was used to predict mutant structures and energetics. The annealing algorithm used in the original method has been modified to continuously collect data on each residue's energy during the random walk, and to use this data to bias the selection of new moves, giving it greater speed. The details of this method will be described elsewhere. For each mutant, the six residue prediction zone (36, 40, 42, 47, 65, 68) was initialized to a completely random conformation, and the structure prediction run until the energy converged; E_{calc} is simply the average energy for the system during the final stage of the prediction algorithm, on a sample size of 1,000 steps. All details of the parameters and computational procedures were absolutely identical for calculation of all 78 mutants.

Potential function and parameters:

Atom	r_0 (Å)	ϵ_0 (kcal mol ⁻¹)
C, S	4.315	0.0738
O	3.553	0.1848
N	3.817	0.4132

Van der Waals interactions were calculated for atom pairs according to

$$E_{\text{vdw}} = \epsilon_0 (r_0/r)^{12} - 2(r_0/r)^6$$

ϵ_0 and r_0 for pairs of unlike atoms were taken to be the geometric means of the values for the two atom types. In addition a torsional potential

$$E_{\text{tor}} = 1.5 \cos(3\chi) \text{ kcal mol}^{-1}$$

was calculated on the following torsions: Ile (χ_1, χ_2), Leu (χ_1, χ_2), Met (χ_1, χ_2, χ_3), Phe (χ_1, χ_2) and Val (χ_1).

* Data from ref. 1 at 26 °C. T_m , melting temperature.

characterized their activity¹. Because their experimental design excludes such uncertain factors as the energetic cost of burying polar or charged groups, electrostatic interactions and even hydrophobicity to some extent (as all five residues are hydrophobic) it addresses in the simplest form the purely structural issues influencing protein activity (for example, which mutations can be packed together to form a stable, active protein). It is this purely structural component which our method computes. We postulate that mutants predicted to have greater internal strain (higher calculated energy) than wild type should be less active because of deformation of the active site, and destabilization of the folded state.

Using the method described above, we predicted structures for a zone of six residues (36, 40, 42, 47, 64 and 68) around the mutations, within a fixed context of the main-chain and other side-chains taken from the 2.5-Å resolution crystal structure⁹, for all 78 sequences studied experimentally. The structure predicted for the wild-type sequence matched the X-ray structure with an r.m.s. error of 0.88 Å (Fig. 1). The calculated energies discriminated active from inactive mutants quite well (Fig. 2a, also see Table 1). In particular, the 10 sequences found experimentally to be fully active at 26 °C (activity grade 5) all had strongly negative E_{calc} (-31.77 to -39.39 kcal mol⁻¹) close to that of wild-type (-35.63 kcal mol⁻¹), whereas the structures of the 22 'inactive' sequences (activity grade 0) were all highly strained, with E_{calc} ranging from -18.95 to 41.47 kcal mol⁻¹. Overall, active mutants clustered in a range -4 to 20 kcal mol⁻¹ relative to the wild-type E_{calc} , whereas inactive mutants ranged from 17 to 75 kcal mol⁻¹ relative to wild-type. The two distributions had relatively little overlap; using the simple cut-off rule 'if $E_{\text{calc}} < 20$ relative to wild-type then active; otherwise inactive' correctly predicted activity versus inactivity with 92% reliability (6 errors out of 78).

For comparison, Fig. 2b reproduces Lim and Sauer's analysis

of the distributions of their active versus inactive mutants over the range of core packing volume, a simple measure often used to forecast internal mutations' viability. Although the distribution of inactive mutants is slightly shifted relative to that of active mutants, their extensive overlap makes volume a poor predictor of activity. Indeed, choosing the optimal cut-off rule of ' $\Delta\text{volume} \leq 3$ then active' is only a marginally better predictor (19 errors out of 78) than simply asserting 'all sequences are active' (22 errors out of 78). Whereas E_{calc} is 22/6 (nearly fourfold better) than this null assertion, volume is only 22/19 (16% better). Furthermore, E_{calc} correctly identifies the inactive sequences at high frequency (17/22), versus volume's dismal 4/22.

Lim and Sauer have isolated and performed thermal unfolding studies on a set of nine mutants, allowing a direct comparison of E_{calc} and experimentally determined stabilities (Fig. 3a). The correlation is surprisingly good. Mutant LLI, for example, has significantly diminished activity (grade 2), yet was calculated to have more favourable energy than wild type, a surprising result. Measured by thermal unfolding, however, it actually is more stable than wild type, as is the other mutant of the set that has lower E_{calc} than wild type (IMV). Overall, lower calculated energy seems to be an excellent predictor of improved stability.

Of the nine sequences, the two containing phenylalanine (FLV and FFI) have anomalously high E_{calc} , lying far off the correlation line running through the other seven data points. In both cases the unusually high energies of the predicted structures are due to collision of the aromatic ring of Phe against side-chains not allowed to move during the calculation (Asn 61, Leu 65, Leu 69). Clearly these are two cases where the assumption that these residues remain fixed is incorrect, not a surprising result in light of these mutants' overpacking. Use of larger molten zones easily identifies such errors, and combined with simple energy minimization may largely correct them.

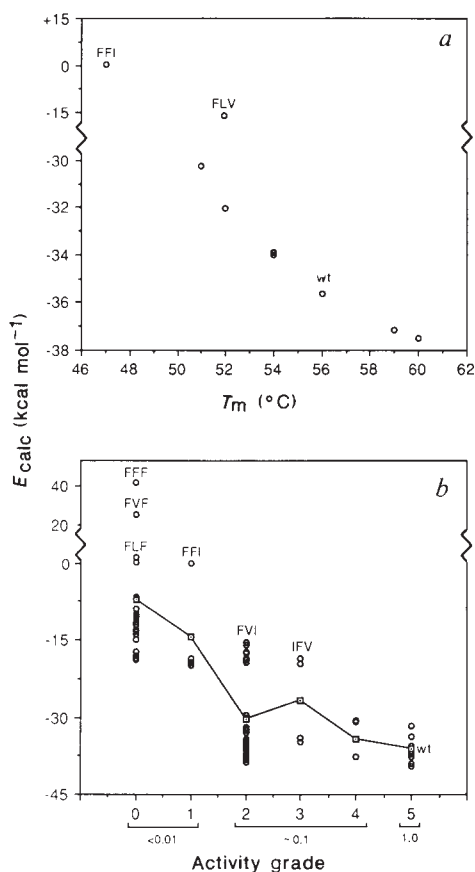
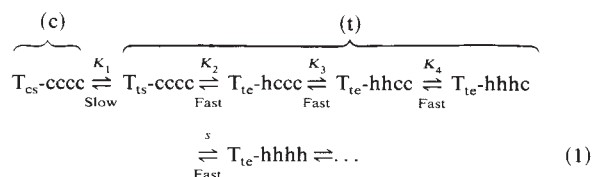


FIG. 3 a, Comparison of predicted energetics with experimentally measured stability. The melting temperatures (T_m) of the nine mutants measured experimentally are plotted against their calculated energy (E_{calc}); the wild-type protein is indicated as wt. The two phenylalanine-containing mutants have anomalously high energies due to collision with side-chains held fixed in the calculation (see text). b, Comparison of predicted energetics with experimentally measured activity. The 78 mutants' activity (measured experimentally) are plotted against their calculated energies. The experimental activity grades 0–5 are defined by a simple plate assay that challenges each repressor mutant-bearing clone with five phage covering a range of different virulence levels. Thus there is no reason to expect a linear relation between the activity grades and true activity. Lim and Sauer report that, among 10 mutants tested, DNA-binding affinity was about 0.1 (relative to wild-type) for mutants in grades 2–4, and <0.01 for mutants in grades 0–1. ○, E_{calc} ; □, grade average.

Initiation of a helix fixes the orientation at four successive single (non-amide) bonds of the peptide backbone. With our template, Ac-Hel₁, two such bonds are constrained to assume helical angles by five-membered rings of the L-proline type, and the third by a synthetically introduced S-CH₂ function that bridges the proline residues (Fig. 1*b*). The resulting constrained derivative of acetyl-L-prolyl-L-proline can be linked by an amide bond to the N terminus of a normal peptide to form Ac-Hel₁ peptide conjugates⁵⁻⁷.



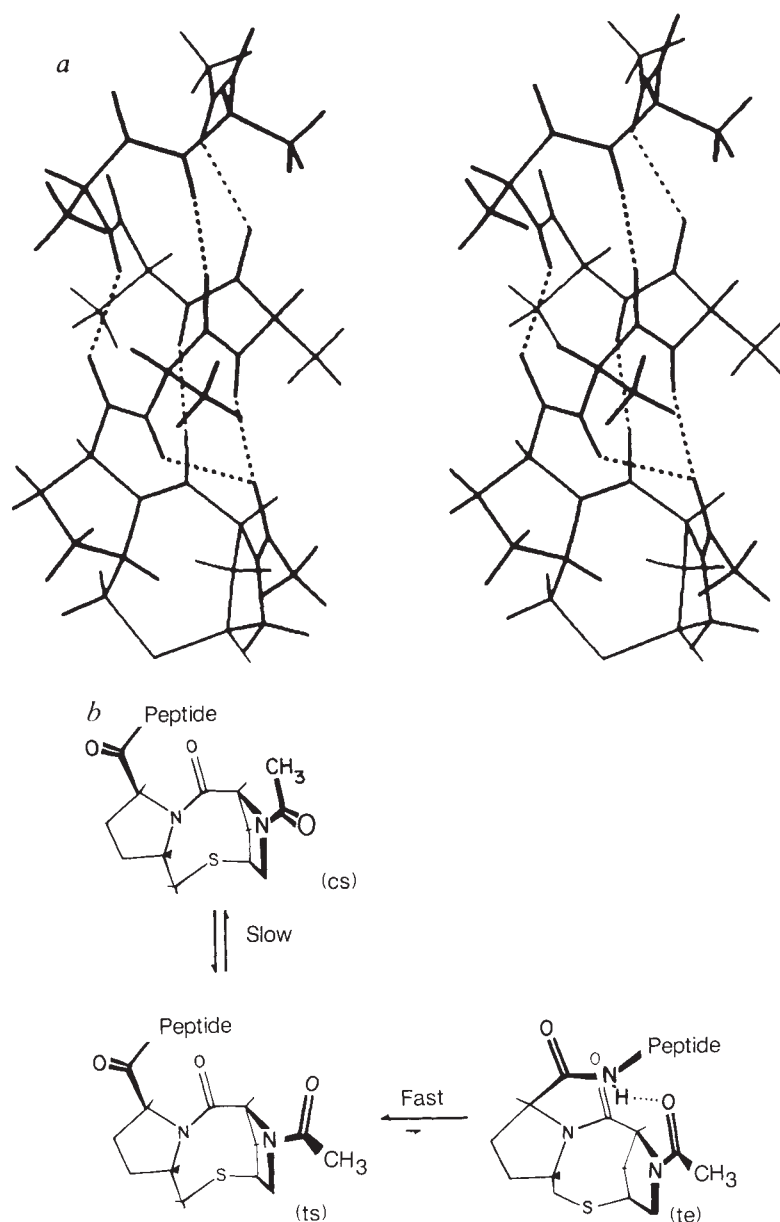
These studies have now been extended to Ac-Hel₁-(L-Ala)₆-

For this method as with other predictive theories, the most fruitful discoveries will come from further interaction and comparison with experimental work¹¹⁻¹⁹. Our tests of the method on these cases obtain similar correlations with experiment, indicating that it may be generally applicable to the stability effects of mutating core hydrophobics. The success of this simple method argues that there is nothing fundamentally mysterious about the interactions that determine stability, nor insuperable obstacles to their computation. $\square$

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FIG. 1 *a*, Stereodiagram of a local energy minimum for an Ac-Hel₁-(L-Ala)₅-NHMe conjugate, with the template region in the (te) conformation shown in detail in *b*. This structure closely approximates an α helix that has bifurcated hydrogen bonding of the 3₁₀- α type (ref. 8) at the first alanine residue. The ¹H NMR spectra of Ac-Hel₁/peptide conjugates in organic solvents are consistent with this conformation⁷, and evidence for helical (t) and random (s) states in water⁶ is given below. *b*, Three of the four possible major conformations of Ac-Hel₁ are observed in solution. The nonnucleating (cs) equilibrates slowly with nonnucleating (ts) and nucleating (te) conformations, which are in rapid equilibrium. Ratios of (t) to (c) populations, obtained by integration of their separate NMR resonances, can be related to helical stability.

METHODS. The structure of Ac-Hel₁-OH in the solid state is established by X-ray crystallography to be (cs). Structures in solution were established by NMR evidence on the basis of chemical shifts, *J* values, and NOE measurements as follows: Ac-Hel₁-OH in CDCl₃; 100% (cs); Ac-Hel₁-OH in D₂O: 60% (cs), 40% (ts); Ac-Hel₁-(L-Ala)₆-OH in CDCl₃ or >10 M% trifluoroethanol in H₂O, 100% (te). The structure in *a* was simulated in vacuum using the Quanta 2.1 version of Polygen CHARMM molecular mechanics software and is consistent with NMR spectra in CDCl₃ and DMSO-*d*₆ (ref. 7). For Ac-Hel₁-(L-Ala)₆-OH in water the following NMR parameters are observed: chemical shifts of NH: (cs) δ 8.2–8.4 (mean δ 8.26), (te) δ 7.6–8.1 (mean δ 7.91); *J*_{αCH-NH}: mean value for central four (te) residues, 5.6 Hz; temperature dependences: (cs) mean value of $-\Delta\delta\text{NH}/\Delta T$, 8.1×10^{-3} p.p.m. K⁻¹, (te) mean value, 4.3×10^{-3} p.p.m. K⁻¹.



OH in water⁶ (see legend to Fig. 1). Distinctive NMR features of random coil and helical peptides in water have been well documented^{9–12}. From magnitudes of chemical shifts and their temperature dependence, as well as coupling constants ³*J*_{H_Nα}, the NH resonances of (t) states of this conjugate can be assigned as helical and those of the (c) states, as random coils.

The nuclear Overhauser effect (NOE) signature of a peptide reflects 1/*r*⁶, where *r* is the weighted average distance between nonbonded hydrogens, and often can be used to define its structure^{13,14}. Helical peptides have strong NOE interactions between adjacent NH functions along the peptide backbone. A ROESY (rotating-frame Overhauser enhancement spectroscopy) experiment¹⁵ in H₂O-D₂O on Ac-Hel₁-(L-Ala-*d*₄)₄-OH reveals its through-space NH connectivities (Fig. 2), which are helical for the (t)-state resonances but undetectable for the (c) states.

Rates of hydrogen-deuterium exchange for backbone NH and circular dichroism (CD) measurements can provide quantitation of helicity in proteins. The presence of nonnucleating (s) states, each with its own template CD ellipticity, along with uncertainties in the length-dependence of 222-nm ellipticities¹⁹, complicates the CD analysis of Ac-Hel₁ peptide conjugates. Preliminary results both of CD and of hydrogen-exchange

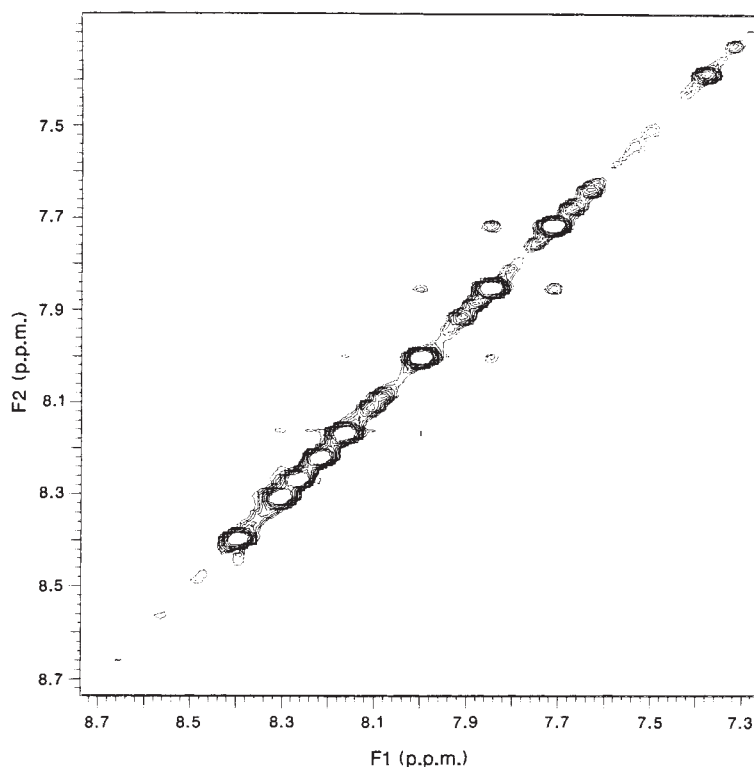
studies are consistent with all other spectroscopic studies. In water the templates (cs) peptide lacks detectable conformational bias and the (te) peptide is an α helix, nucleated outward from the amino-acid residue at the template junction.

The three structural series Ac-Hel₁-(L-Ala)_{*n*}-OH, *n* = 2–7, Ac-Hel₁-(L-Ala)_{*n*}-Lys-NH₂, *n* = 3–8 and Ac-Hel₁-Gly-(L-Ala)_{*n*}-Lys-NH₂, *n* = 2–8, all show a temperature-independent, progressive increase in (t)/(c) ratio as the peptide length *n* is increased. Following an analysis similar to that used by others for helix-coil transitions¹, we use this length dependence to calculate *s* values for alanine in water (Fig. 3). Two different structural series yield an *s* value of 0.95 ± 0.15 which is consistent with 1.06 reported by Scheraga³.

Measurement of the position dependence of the disruptive effect of a helix-breaking glycine residue allows an independent evaluation of *s*. The relative (t)/(c) values in water for the six pairwise-permuted Ala-Gly isomers of Ac-Hel₁-Gly-(L-Ala)₅-OH are shown in Fig. 4. The change in (t)/(c) with increase in separation between template and glycine residue is uniquely consistent with frayed helices characterized by an *s* value close to 1.0.

The helices observed in short peptides by the Baldwin group and others are both initiated within and propagated by the

FIG. 2 Section of the NH resonance region of a 500 MHz ROESY spectrum^{15,16} of Ac-Hel₁-(L-Ala)₄-OH in 9:1 H₂O-D₂O, pH 2.1, 25.0 °C. Both positive and negative peaks are displayed without discrimination. The four alanine NH functions of the (cs) state appear along the diagonal above 8.15 δ and reveal no NH-NH connectivities, consistent with a random-coil structure. The four NH functions of the (te) state appear along the diagonal at δ 7.70 (NH 1), 7.83 (NH 2), 7.99 (NH 3) and 8.15 (NH 4) and reveal helical $d_{\text{NH}}(i, i+1)$ NOE connectivities that decreased in intensity with distance from the template, the pattern expected for a substantially frayed helix. METHODS. The spectrum was acquired on a Varian VXR500S spectrometer with 2.0 s presaturation of water and a 3.3 kHz pulsed spin lock¹⁷ during the 350-MHz mixing time. The 30° mixing pulse was preceded and followed by a 90° pulse for resonance offset compensation. For each hypercomplex data set¹⁸, 128 scans of 405 increments were collected over a 4,900-Hz sweep width, zero-filled to a 2K $\times$ 2K matrix and transformed with gaussian weighting in both dimensions.



peptide. The helicity of a short peptide usually can be modelled by a series of pairs of s and σ values that vary inversely, with σs^n a constant¹. We conjecture that Scheraga's s values apply generally to helical peptides, but that σ values can be sequence-dependent and can substantially exceed the expected range of 10^{-3} – 10^{-4} , resulting in detectable helicity for selected short peptides.

An initiation step results in loss of conformational freedom

and must exhibit a negative ΔS° . Consequently a σ value near 1 (ΔG° close to zero) must be associated with a negative ΔH° . The temperature dependence of helicity that has been observed by Baldwin for short helical peptides^{4,20} may thus reflect anomalously high σ values.

A high σ that is sequence-dependent could result in detectable helix structure in a very short peptide region. There is at least one documented example of this type. Wright and coworkers

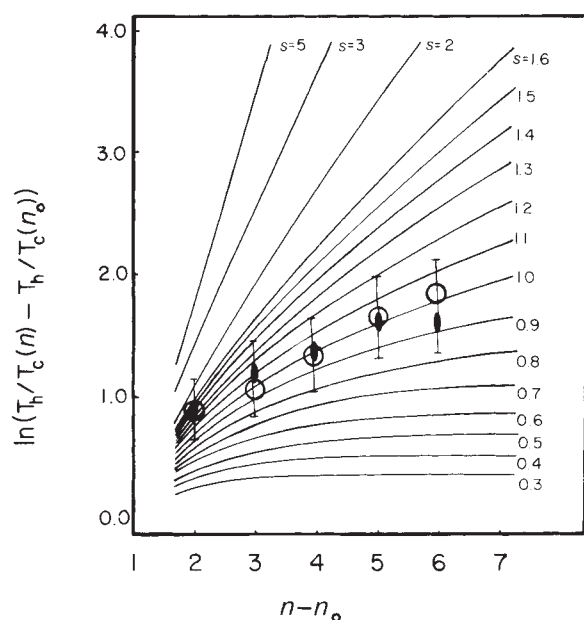


FIG. 3 Assignment of s from the length dependence of the (t)/(c) ratio in 9:1 H₂O-D₂O at 23 °C, pH 1–2. Data are represented as solid circles for Ac-Hel₁-(L-Ala)_n-Lys-NH₂, $n=4$ –8 with n_0 defined as 2 and the derivative, $n=3$ as reference, and as open circles for Ac-Hel₁-Gly-(L-Ala)_n-Lys-NH₂, $n=3$ –7 with n_0 defined as 1 and the derivative, $n=2$ as reference.

METHODS. The (t)/(c) values were measured as ratios of selected ¹H NMR peak areas. Lysinamide termini are added to increase solubility. Identical (t)/(c) ratios of 1.85 were observed for Ac-Hel₁-(L-Ala)₅-Lys-OH and Ac-Hel₁-(L-Ala)₆-OH. The calculated curves of Fig. 3 are fitted to integer values for a given s and $n - n_0$, from $\ln(1 + s + \dots + s^{n-n_0-1})$, $n - n_0 = 2$ –7.

$$\begin{aligned}\chi_m &= (t)/(c) \\ &= A + B(1 + s + s^2 + \dots + s^{m-4}) \\ A &= K_1 + K_1 K_2 + K_1 K_2 K_3 + K_1 K_2 K_3 K_4; \\ B &= K_1 K_2 K_3 K_4 s\end{aligned}\quad (2)$$

where m = the number of amide NH in the peptide backbone

$$\begin{aligned}\ln(\chi_m - \chi_i) &= \ln(1 + s + \dots + s^{m-i-1}) \\ &\quad + \ln K_1 K_2 K_3 K_4 s^{i-2} \quad 3 < i < m\end{aligned}\quad (3)$$

Equation (2) can be derived from (1) with the assumption that constants K converge to s after the third helical residue¹. The following analysis eliminates coefficients A and B of (2). A peptide conjugate, for example Ac-Hel₁-(L-Ala)₃-Lys-NH₂, is selected as the reference, and its χ_i value is subtracted from each χ_m for members of a series of higher oligomers. Values $\ln(\chi_m - \chi_i)$ are calculated. From (3) these equal a sum of two terms, the first dependent only on s and m , and the second constant for all series members. A graph of $\ln(\chi_m - \chi_n)$ vs $n - n_0$ is superimposed on the calculated curves of Fig. 3, and vertical displacement is used to find the optimal fit of data. Alternatively the block of equations (2) obtained from an assumed s and a set of experimental (t)/(c) for a range of m are subjected to linear least squares analysis to give best A and B with the error term $\sum (\chi_i - \chi_{i\text{calc}})^2$; s is then varied to minimize this term. Values of 0.95 and 0.90 were found for the series Ac-Hel₁-(L-Ala)_n-Lys-NH₂ and Ac-Hel₁-Gly-(L-Ala)_n-Lys-NH₂.

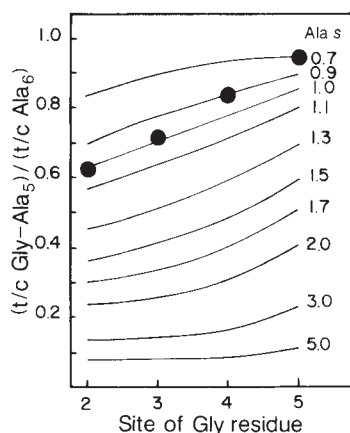


FIG. 4 Calculation of s for alanine from an analysis in 9:1 $\text{H}_2\text{O}-\text{D}_2\text{O}$ at 23 °C, pH 1 of the variation of the $(t)/(c)$ ratio with glycine position for the Ac-Hel₁-Gly-(L-Ala)₅-OH isomer series. In the series glycine replaces each alanine residue in turn in the template conjugate. Data points are calculated as the $(t)/(c)$ ratio for each Gly-Ala₅ position isomer divided by the $(t)/(c)$ value for Ac-Hel₁-(L-Ala)₆-OH. The sensitivity of the template to the disordering effect of a glycine drops off rapidly with distance for strongly frayed templates in which little helical structure persists at three to four residues from the template (s values near 1.0). The sensitivity is expected to be nearly site-invariant for robust helices (s values > 2). METHODS. Lines of the graph are calculated from equation (4), obtained from equation (2) with the approximation that K_3 and K_4 are set equal to s .

$$(t)/(c) = K_1 + K_1 K_2 + K_1 K_2 s(1 + s(1 + s(1 + s(1 + s)))) \quad (4)$$

Coefficients $(K_1 + K_1 K_2) = 0.76$ and $K_1 K_2 = 0.24$ were obtained for Ala oligomers by least squares analysis as described in Fig. 3. Estimated $(t)/(c)$ ratios for a series of Gly-Ala₅ position isomers were obtained for an assumed Ala s value by setting one particular s value in equation (4) equal to a Gly value of 0.4; these ratios were divided by the values for Ac-Hel₁-Ala₆-OH calculated from equation (4) with all s values equal. Analysis of Ac-Hel₁-Gly _{n} -OH series by the method of Fig. 3 yields a range for the Gly s value of 0.3–0.6.

have observed NMR properties consistent with weak helicity at 5 °C in water for a pentapeptide sequence of an 18-amino-acid C-terminal fragment of myohaemerythrin²¹.

The magnitudes and ranges of s and σ values define the intrinsic nature of helical states in solution. These first results for templated peptides in water provide evidence for separation of the propagation constant s from effects of initiation. Further experiments that permit independent characterization of the initiation constant σ will be required to resolve this issue. □

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A human and bovine serum alternative and a microbalance that cuts down on unwanted temperature fluctuations and vibrations by separating the weighing cell from the control unit — new product news from Switzerland.

MEASURING 390 × 330 × 98 mm, the new **rotary shaker** from Infors called Labotron, is designed to be small enough to fit into incubators and humidification cabinets (*Reader Service No. 100*). The instrument's shaking table is built as a universal tray with a grid of holes onto which it is possible to screw any size of clamp and swivelling test tube rack: a typical tray capacity for the Labotron is 12 × 250-ml or 5 × 1,000-ml flasks. Labotron will operate in temperatures of up to 60 °C and features a shaking speed range of 40–400 r.p.m. A powerful motor drives the shaking table, says Infors. The fly wheel is arranged centrally and designed as an eccentric guide, carrying the shaking table, which is mounted on bearings to ensure an exact, horizontal rotary shaking motion. Its big brother, the Aquatron shaking waterbath, features a transparent cover to allow a gas-tight closure and clear visibility of the flasks inside. The anti-corrosive plastic housing and bath interior are impact-resistant, inert to most chemicals and easy to clean, the company says. Operation of the Aquatron at below ambient temperature can be achieved with the optional cooling coil.

Salvis has introduced the Biocenter 2001 **CO₂ incubator**, which is designed to provide stable culture conditions for the following areas of research: retroviral research, cancer and tumour research, research on brain and nerve cells, immunology, toxicity testing, and cell and molecular biology (*Reader Service No. 101*). The Biocenter 2001 uses a microprocessor to monitor important cul-

ture parameters such as temperature, CO₂ and humidity. The IR system used to measure CO₂ content is unaffected by changes in temperature or humidity, in contrast to conventional heat conductivity systems, Salvis says. During operation, three filters and pre-set air circulation are designed to ensure germ-free conditions, even when the door is open. As a safety measure, visual and audible alarm systems warn of deviations in temperature, CO₂ and humidity. All parameters are displayed automatically in 5-second intervals. In addition, parameters are stored in case of power failure. The electropolished interior of the Biocenter 2001 facilitates cleaning; the shelves, water dish and sensor module can be removed for autoclaving. An optional O₂ control unit is available for cell cultures that require a controlled concentration of both O₂ and CO₂. This option provides optimal cultivation conditions for bone marrow stem cells, nerve and brain cells, Salvis says.

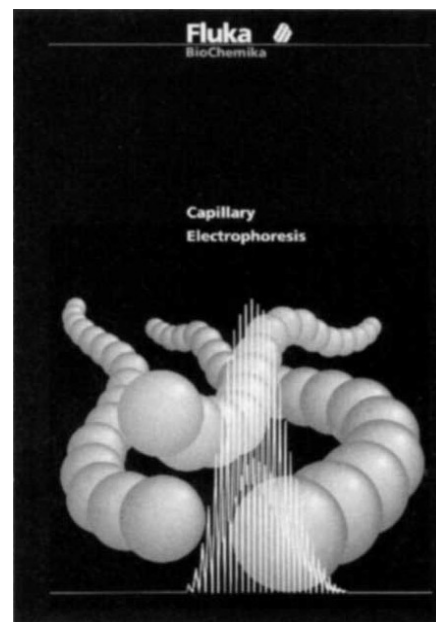
Shelf stockers

To facilitate the design of antiviral agents, Bachem Bioscience has developed a **screening kit** that includes recombinant HIV protease, a HIV substrate and a HIV inhibitor (*Reader Service No. 102*). The company says the recombinant HIV protease was prepared by inserting a synthetic gene into the pET vector 3' to the T7 RNA polymerase promoter and this construct was cloned into an *Escherichia coli* strain that is a lambda lysogen for T7 RNA polymerase. The overexpressed protein accumulated intracellularly in an insoluble form, resulting in inclusion bodies in the cytoplasm, Bachem says. These protein aggregates were then solubilized and purified by conventional chromatography. The monomeric HIV protease was refolded to the active dimer by a dilution folding procedure, Bachem says. In addition to the above, HIV-1 transactivating regulatory peptide, HIV-1 regulator of expression of virion proteins and HIV reverse transcriptase will be available shortly.

To overcome some of the shortcomings associated with bovine or human serum albumin, Pentapharm has developed a novel **polypeptide fraction of purified dermal collagen of porcine origin** called Prionex (*Reader Service No. 103*). Prionex, which the company claims exhibits

protein stabilizing properties that are at least equal to that of BSA, is available as a sterile, pyrogen-free aqueous solution containing 10 per cent solids. It is free of lipids, polysaccharides, nucleic acids or stabilizing agents. The manufacturing process of Prionex — heating at 145 °C and sterilization through a 0.2-μm membrane — inactivates common viruses, bovine spongiform encephalopathy and Scrapie-like infective agents, Pentapharm says. Prionex is available in 20-, 100- and 500-ml quantities and should be stored at room temperature.

To meet the growing demand for **reagents for high performance capillary electrophoresis (HPCE)**, Fluka BioChemika has developed a series of ready-to-use reagents, which includes 21 buffer solutions covering the pH range of 2.5 to 11, two capillary wash solutions and special HPCE-grade water (*Reader Service No.*



Multipurpose CO₂ incubator from Salvis.

Fluka's ready-to-use HPCE reagents.

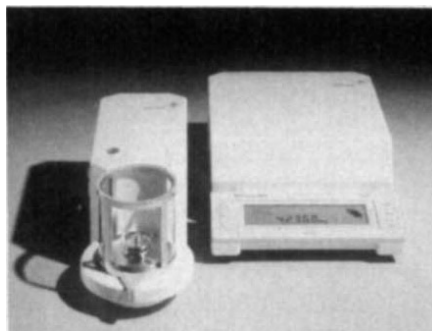
104). The company claims that the buffers are free of insoluble impurities as they are filtered through a 0.2-μm filter membrane after production, exhibit minimal absorption over a wide wavelength range, are virtually free of fluorescent impurities, and are tested for application. Each buffer is sold in 100- and 500-ml quantities, costing DM19 and DM47.10 (Germany), respectively. Water for HPCE is sold in 1-litre quantities and costs DM22.40. The two capill-

ary wash solutions, sodium hydroxide and hydrochloric acid solution, are sold in 500-ml quantities each costing DM16.10. Fluka also offers a broad range of modifiers of high purity (for example, various types of surfactants) and fluorescent dyes for the detection of the analytes.

A selection of **ELISA reagents for diagnosing plant diseases** of agricultural crops, such as potatoes, grape vine, fruit trees, cereals, vegetables and ornamentals is now available from Bioreba (*Reader Service No. 105*). The reagents, which come in kit form, include purified polyclonal and/or monoclonal antibodies and enzyme conjugates for either 1,000 or 5,000 tests. As optional extras, Bioreba will supply all the buffers, solid-phase supports and positive and negative controls for use with the kits. Laboratory equipment that is specially designed to tackle plant tissue extraction, homogenization and liquid handling is also available. Bioreba also offers a consulting service to help researchers set up an ELISA-testing laboratory. Training can be obtained by visiting Bioreba's facility in Switzerland or, alternatively, a Bioreba representative can visit your research establishment.

Weights and measures

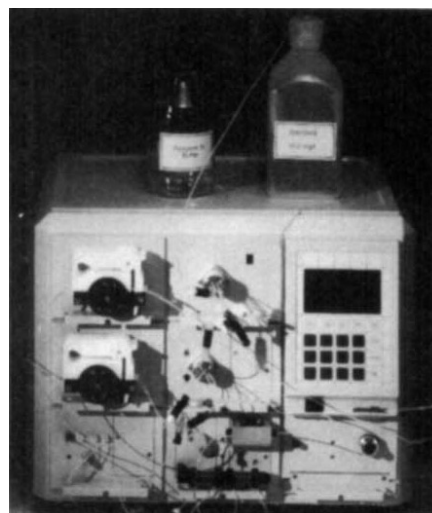
Two features catch the eye with the new MT5 microbalance from Mettler: separation of the weighing cell from the control unit and the new design of the automatic draft shield (*Reader Service No. 106*). With the former, disturbing heat effects in the weighing chamber are reduced dramatically and so acclimation of the sample in the chamber is unnecessary, Mettler says. This set up also keeps vibrations to a minimum. The small-sized cylindrical draft shield is intended to improve the thermal equilibrium. The MT5, which has a weighing range of 1 μ g to 5 g, is equipped with FACT (full automatic calibration technology), which calibrates the instrument automatically as soon as built-in sensors detect a change in



MT5 — making light work of weighing.

ambient conditions. As the MT5 is calibrated and linearized at the maximum load of 5 g, maximum precision over the entire weighing range is assured, Mettler says. Apart from loading/unloading the sample on the weighing pan, all manipulations — opening/closing the weighing chamber and taring — are carried out using the keypad.

User-friendly microprocessor control and the capability for modular expansion are features of the new **ASIA flow-injection analyser** from Isamec (*Reader Service No. 107*). Flow injection analysis



Modular means with Isamec's FIA system.

(FIA) automates an analysis by automating the solution handling of the analytical reaction. With FIA, tasks like dosing, moving and mixing solutions can be performed automatically with pumps and valves. Sample and reagent react in specific manifold parts like mixing coils, diffusion chambers, degassers, temperature-controlled mixing coils (heater) or packed-bed reactors (reaction column). At the heart of the system is the master rack, which includes the control module with screen, keyboard and ASIA software, a power supply, three analog-to-digital converters, a serial interface, seven slots for plug-in modules and a socket for external ASIA modules. From the keyboard, the operator can control all of the ASIA system components and

handle data acquisition from up to three detector signals through the three high-resolution analog-to-digital converters.

Dendro-2003 represents the third generation of data acquisition instruments from Walesch Electronic that is designed for **dendrochronological density analysis** using X-ray technology (*Reader Service No. 108*). It is particularly suitable for the densitometrical analysis of structurally heterogeneous objects in the 50- μ m to 30-cm range. Samples can be enlarged 10, 25 or 50 times (with the option for other enlargement factors as well) as a flicker-free, high-resolution image that is projected upon a 300-mm round screen, Walesch says. In the screen are built-in photosensors, which can be adjusted in angle to the growth-ring boundaries and allow a density measurement on film (slit width 4 μ m or 50 μ m, slit height 0.14–1 mm). The tree-ring definition will largely be provided automatically by the system. Manual tree-ring definition and corrections are possible at any time and can be seen on the screen. After digitization of the density values, the results may be displayed in graphic form on the computer display. It is possible to correlate this curve with other single curves or chronologies. The instrument can be used to reconstruct local and worldwide climatological conditions over thousands of years and provide evidence of natural and man-made environmental changes in forests, lakes and oceans, says Walesch.

The Dosimat Trio from Metrohm, which includes the 665, the 725 and the 715 **dispensing devices**, is designed to meet all types of dispensing needs in applications such as preparative work, HPLC, voltammetry or titrations (*Reader Service No. 109*). The 665 Dosimat is a multipurpose instrument that can be used for titration purposes, as an electronic dropping funnel, and for pipetting, dilution and dispensing. The standard equipment includes an RS232C interface for the attachment of a balance, printer or PC. An analog output option allows monitoring of kinetic processes. The 725 Dosimat performs all the basic functions of the 665, its big brother. It can dispense, dilute and, with an optional accessory, pipette. Metrohm says the 725 is particularly useful as an automatic volumetric flask for the preparation of standard solutions in, for example, voltammetry, photometry, HPLC or spectroscopy. The user weighs out an approximate amount of substance to be dissolved, enters the weight, and the 725 Dosimat then calculates the required amount of solvent and adds it. For simple titrations, the results can be calculated automatically. Completing the trio, is the 715 Dosimat, which is useful for manual titrations. □

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